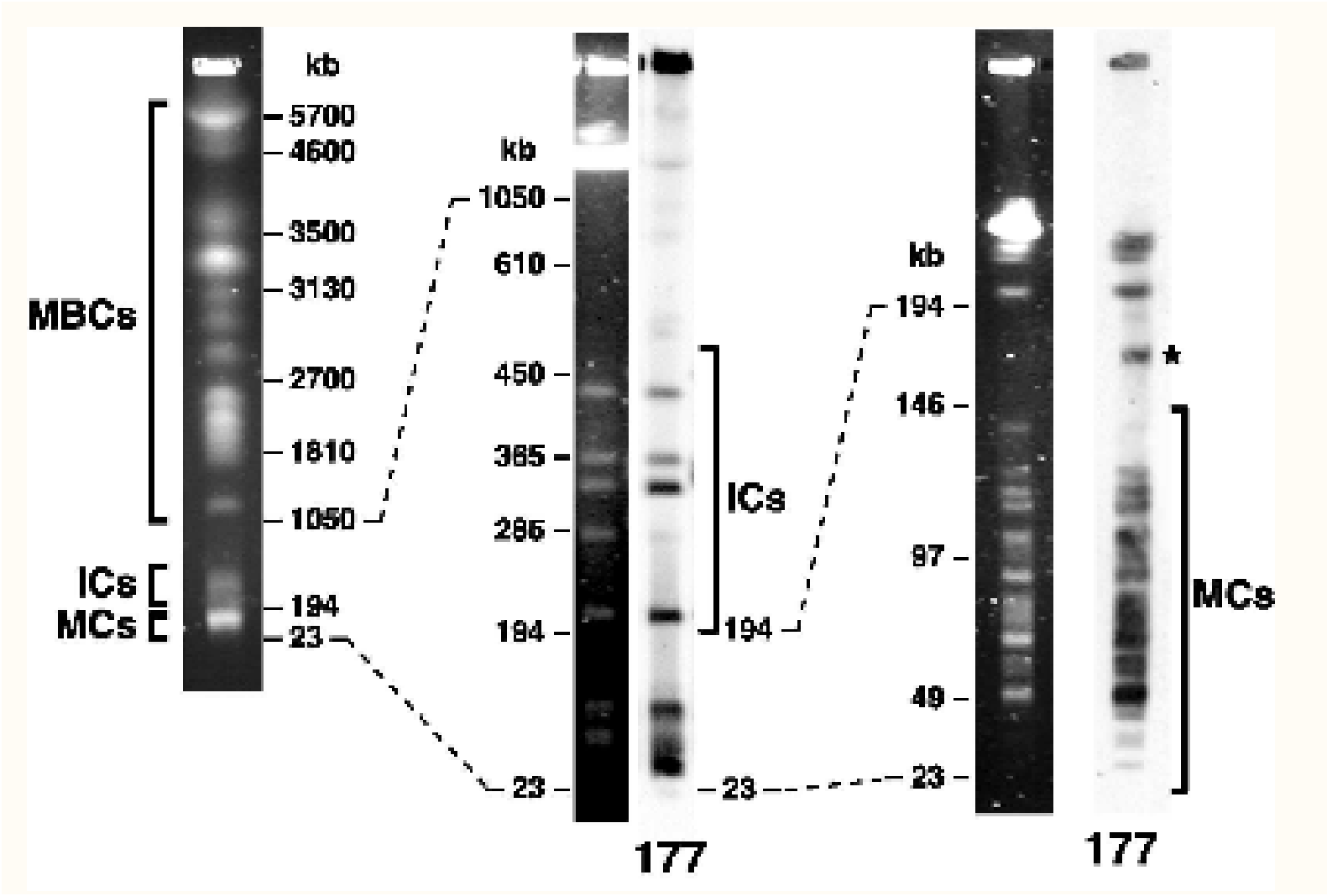
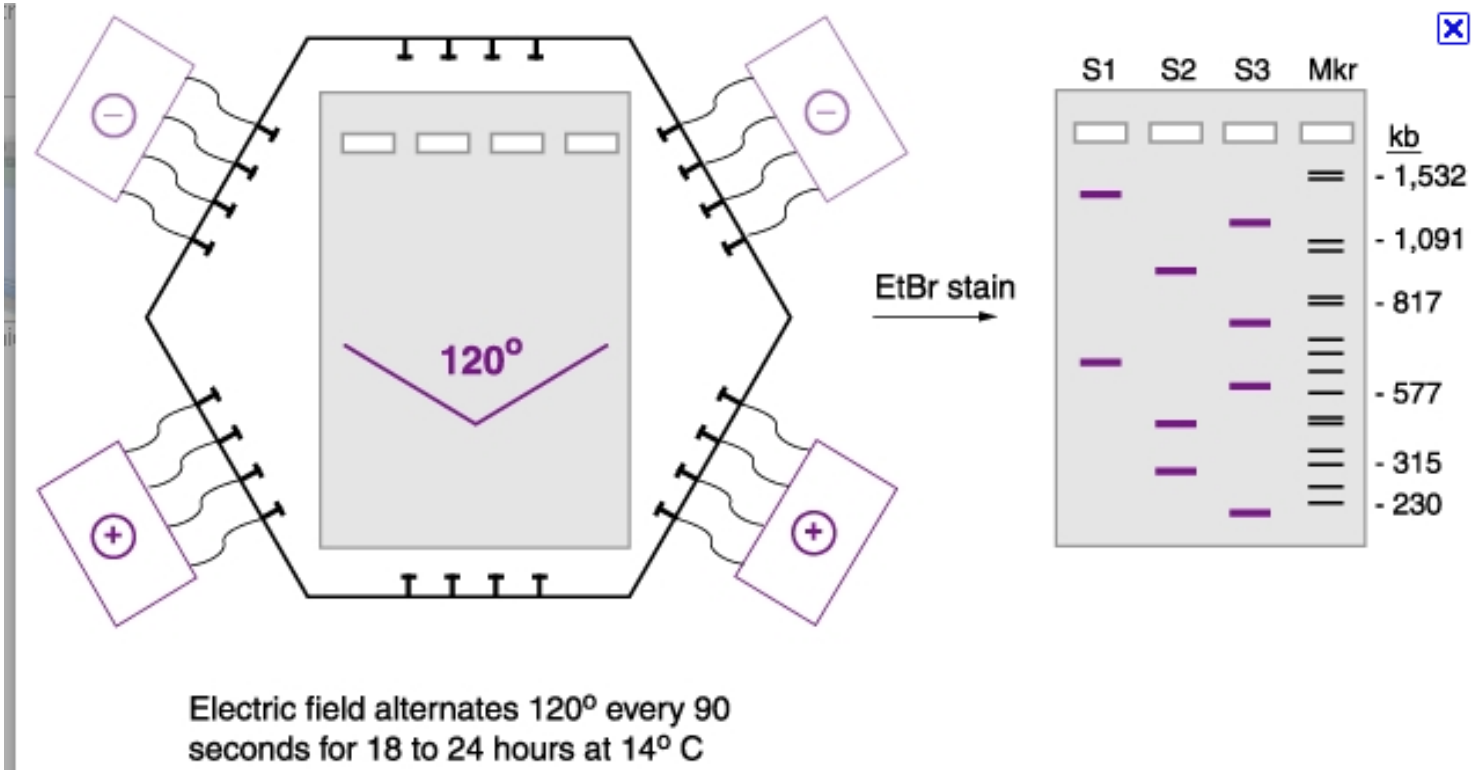
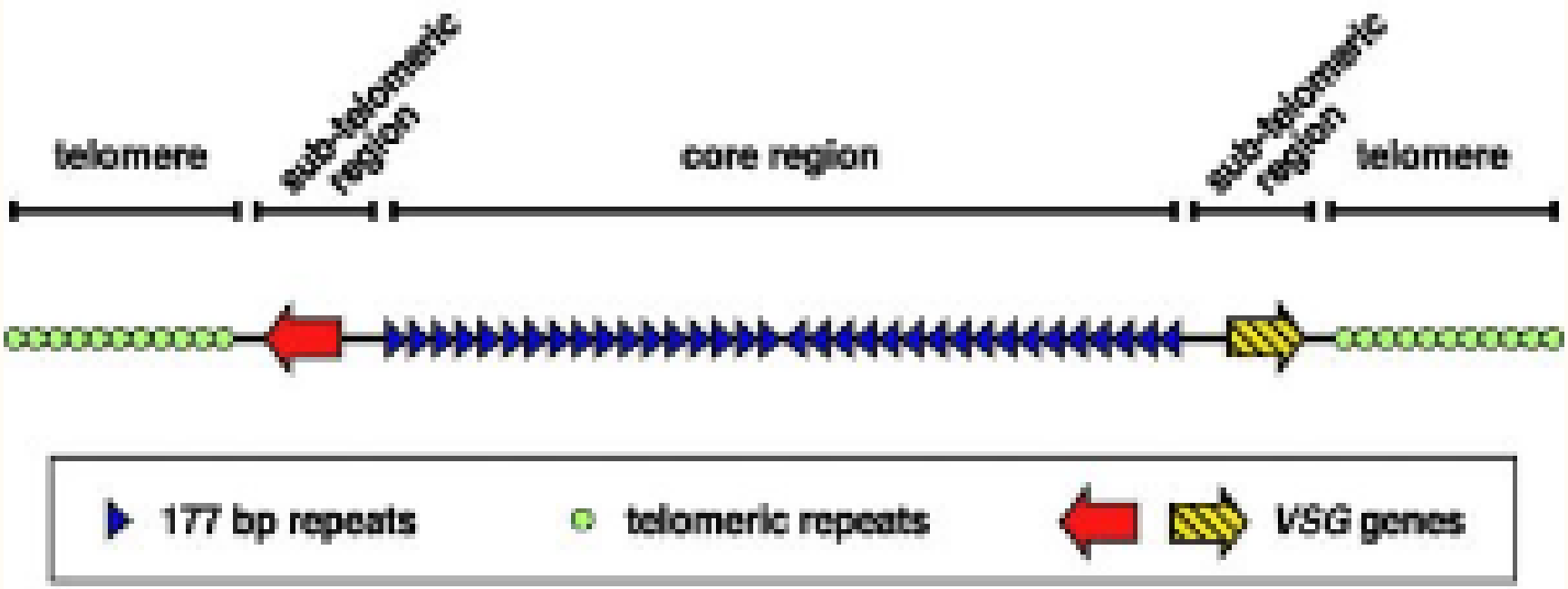
The karyotype of *Trypanosoma brucei* demonstrated by pulsed-field gel electrophoresis of whole-chromosome sized DNAs. The chromosomes can be divided into 3 classes: megabase-sized chromosomes (MBCs), intermediate-sized chromosomes (ICs) and numerous minichromosomes (MCs)



Pulsed Field Gel Electrophoresis

David Schwartz in 1982 suggested that periodically changing the orientation of the electric field would force DNA molecules in the gel to relax upon the removal of the first field and elongate to allign with the new field. This process is size dependent. Chromosomal DNA must first be embedded in agarose plugs and these plugs are treated with enzymes to digest the proteins, leaving behind the naked DNA. The plugs are then cut to size, loaded into the wells of the gel and sealed into place with agarose.

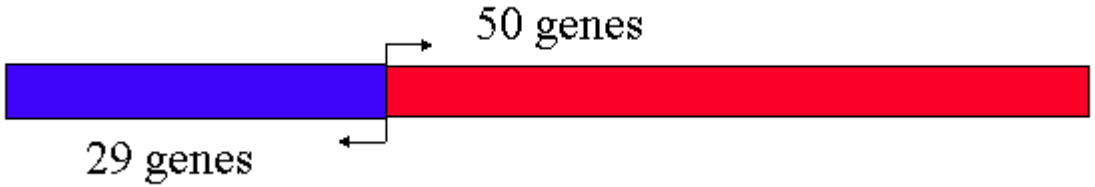




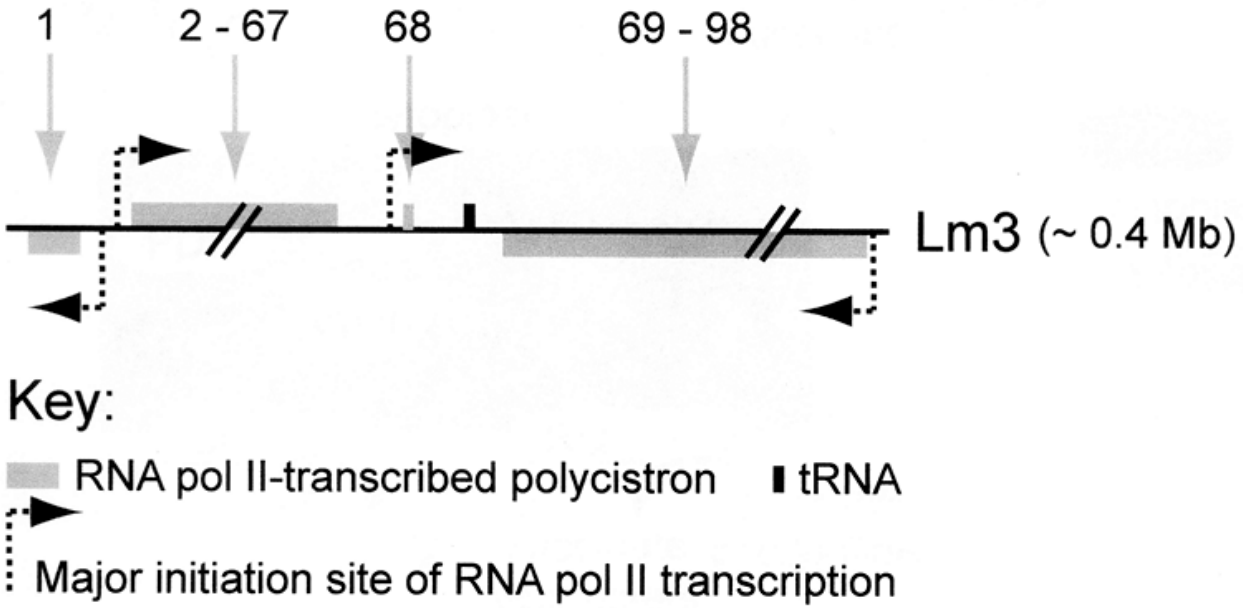
A cartoon illustrating the structure of the MCs showing the repetitive palindrome common to all the small chromosomes.

Polycistronic Transcription

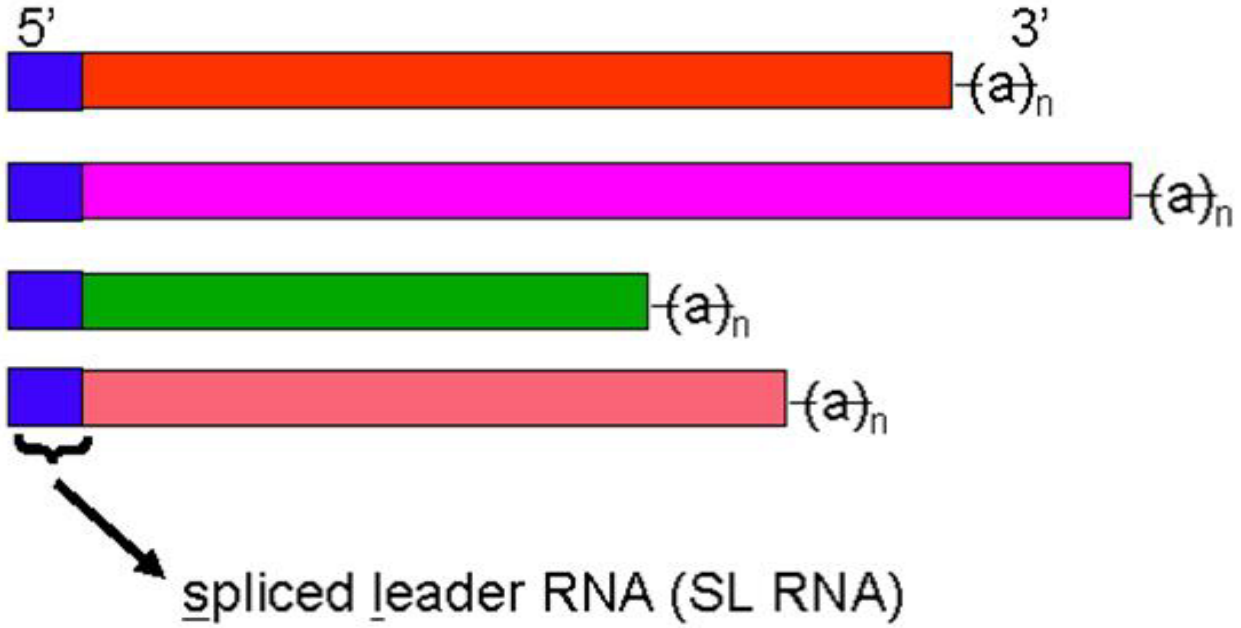
Chromosome 1 of *T. brucei*



Chromosome 3 of *T. brucei*



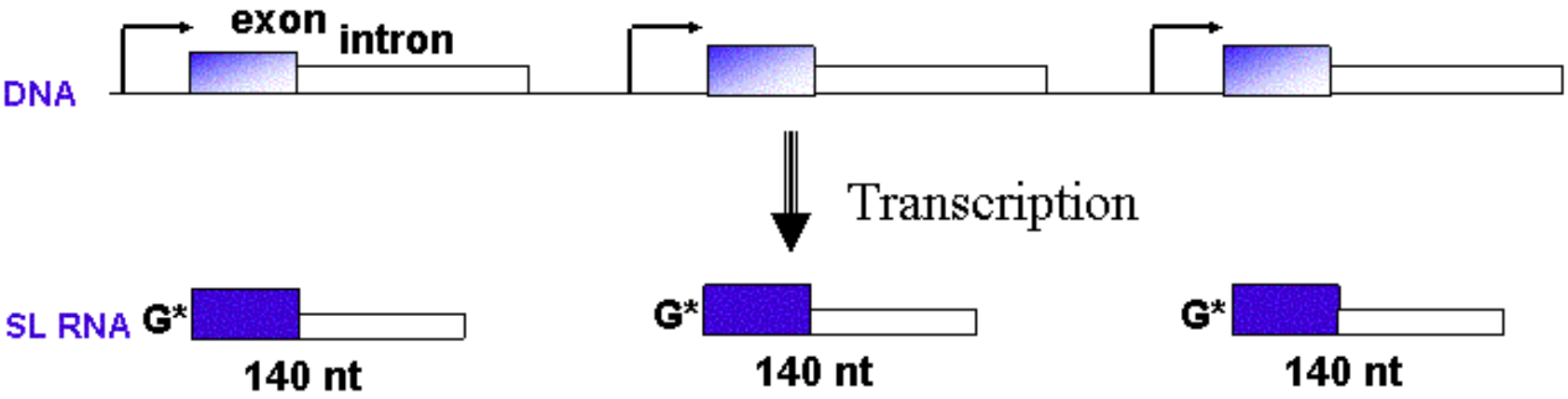
Every mRNA has a 39 nt “spliced leader” or “miniexon” at the 5’ end



This is added post-transcriptionally by “trans-splicing” of a Spliced Leader or SL RNA

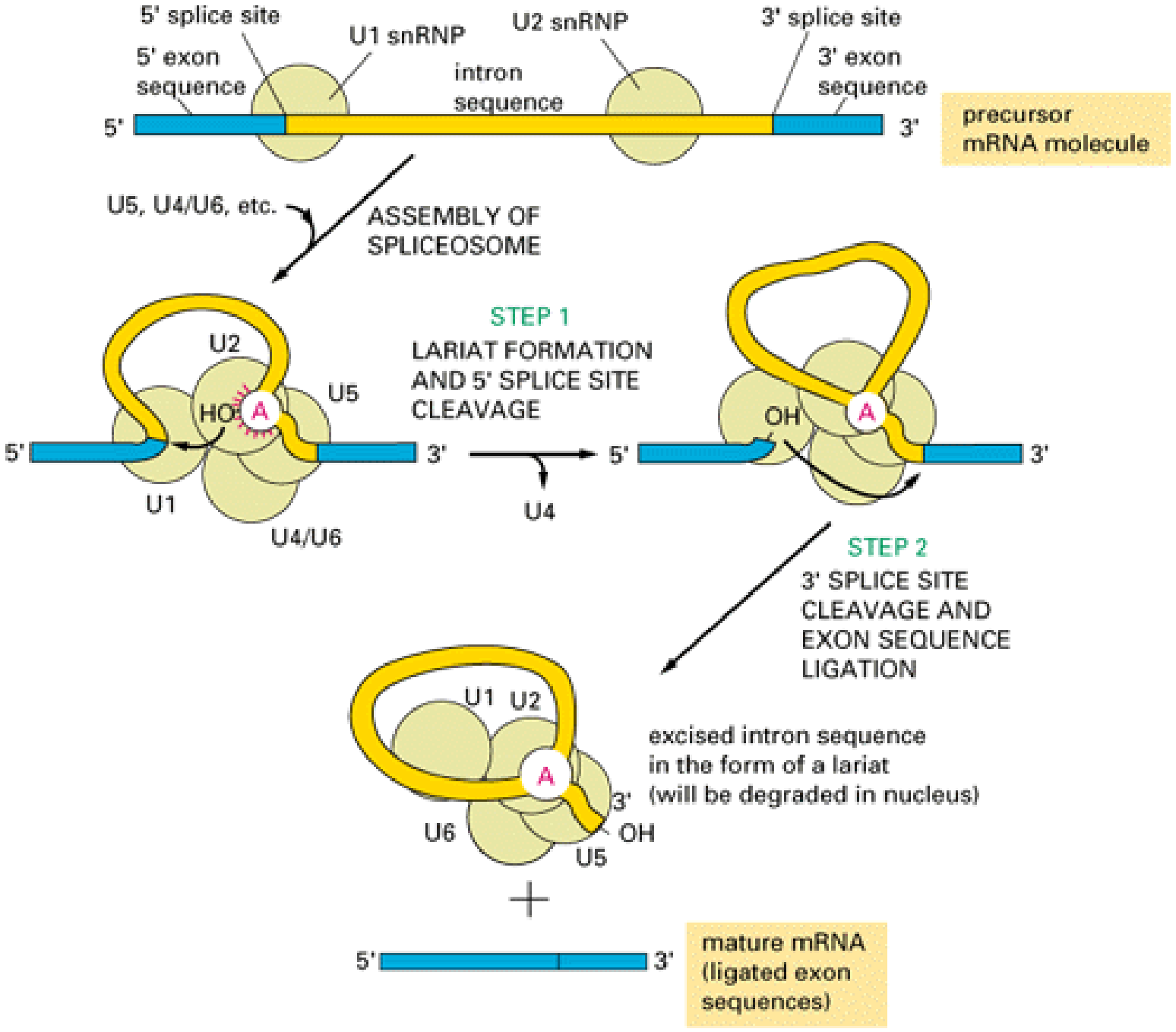
The SL RNA is Transcribed Independently of Protein-coding Genes

- The SL RNA genes - a tandem array of 50-100 copies per cell

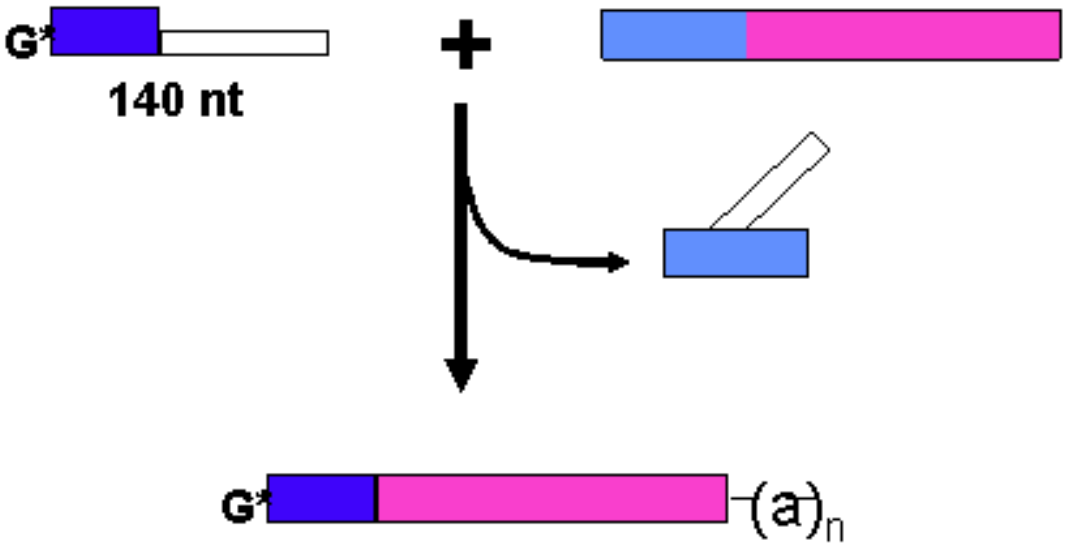


David Campbell and Nancy Sturm and undergraduate student (Shereese Alexander)

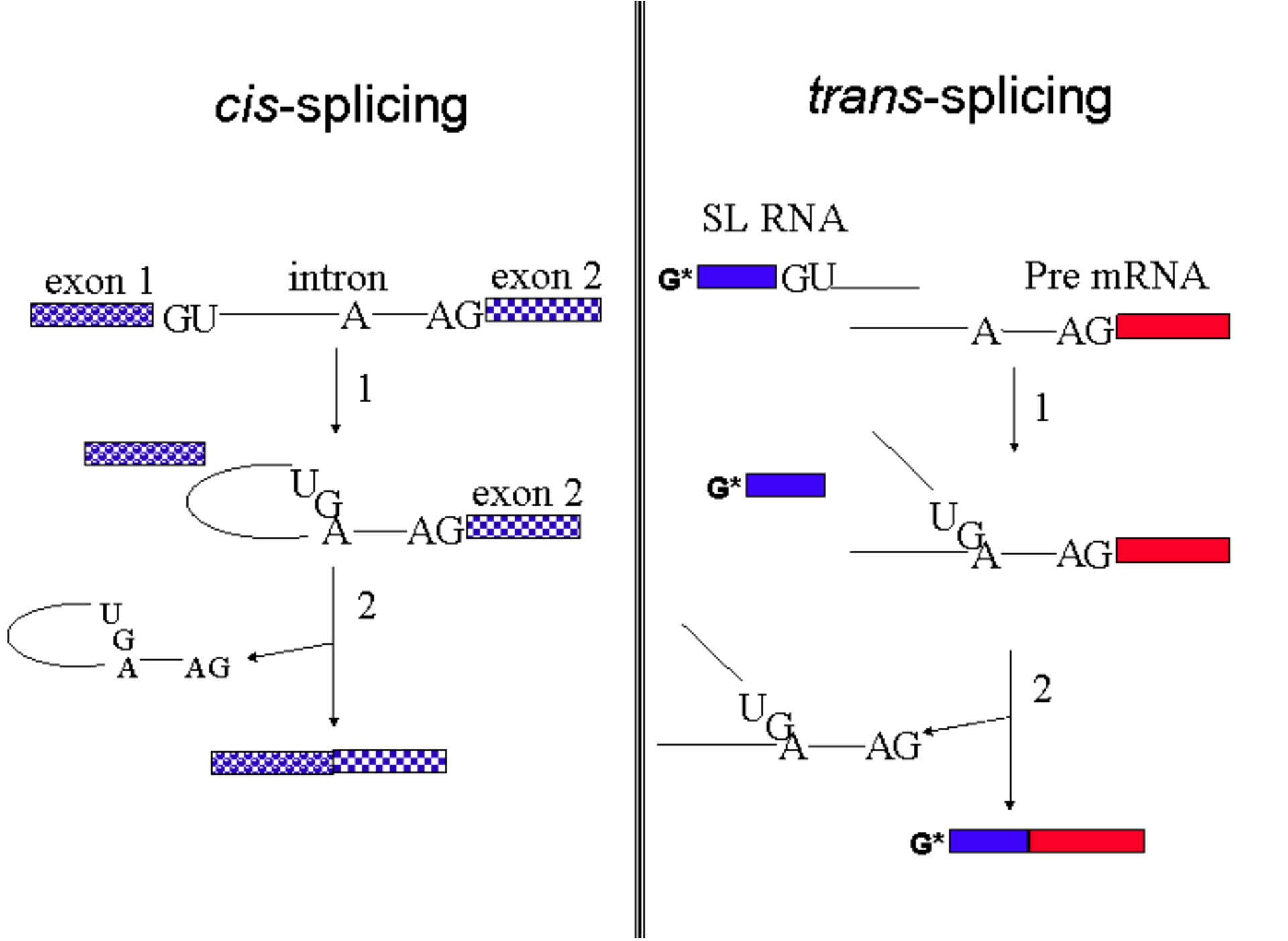
This transcription uses RNA Polymerase II, as does transcription of mRNAs


5' splice site
U1 snRNP
U2 snRNP
3' splice site
5' exon sequence
intron sequence
3' exon sequence
5'
3'
precursor mRNA molecule
U5, U4/U6, etc.
ASSEMBLY OF SPLICEOSOME
STEP 1
LARIAT FORMATION AND 5' SPLICE SITE CLEAVAGE
U2
U5
HO
A
U1
U4/U6
U4
OH
STEP 2
3' SPLICE SITE CLEAVAGE AND EXON SEQUENCE LIGATION
U1 U2
excised intron sequence in the form of a lariat (will be degraded in nucleus)
U6
U5
mature mRNA (ligated exon sequences)

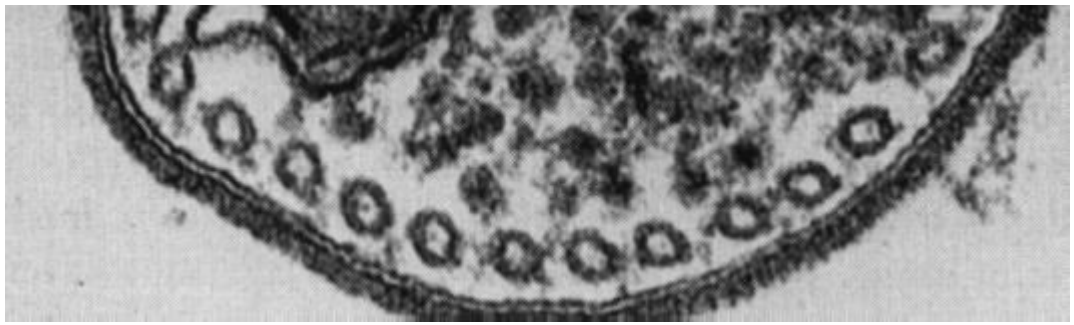
SL RNA exon is *trans*-spliced to protein coding mRNA



- Why?
  - 5' 7mG cap to mRNA
  - stability of mRNA
  - translation


cis-splicing
exon 1
intron
exon 2
GU
A
AG
1
U
G
A
AG
exon 2
2
U
G
A
AG
trans-splicing
SL RNA
G*
GU
Pre mRNA
A
AG
1
G*
U
G
A
AG
U
G
A
AG
2
G*

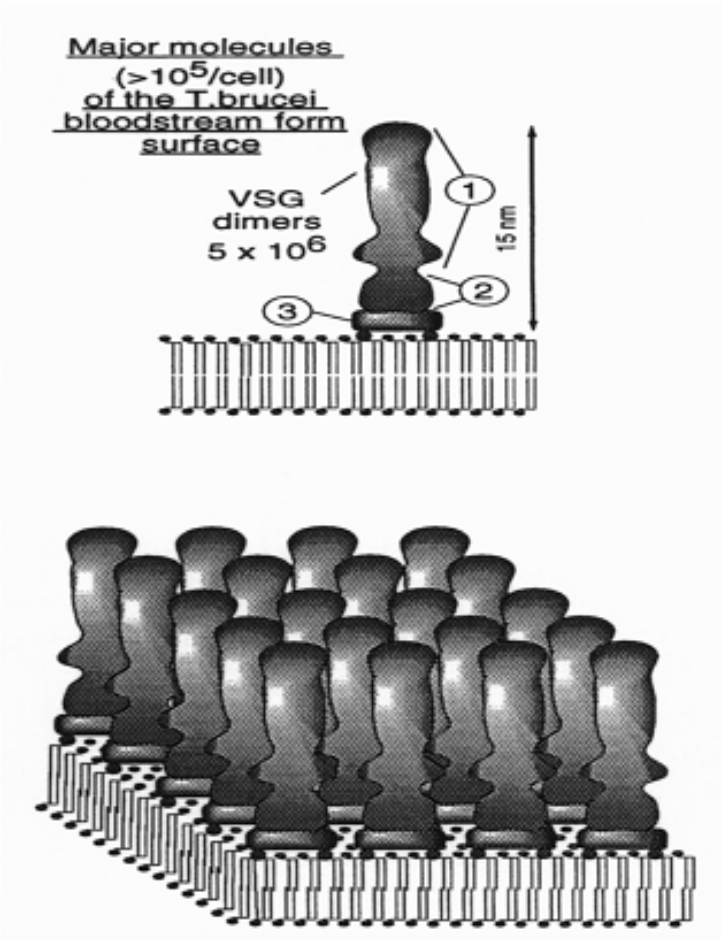
Variant Surface Glycoprotein



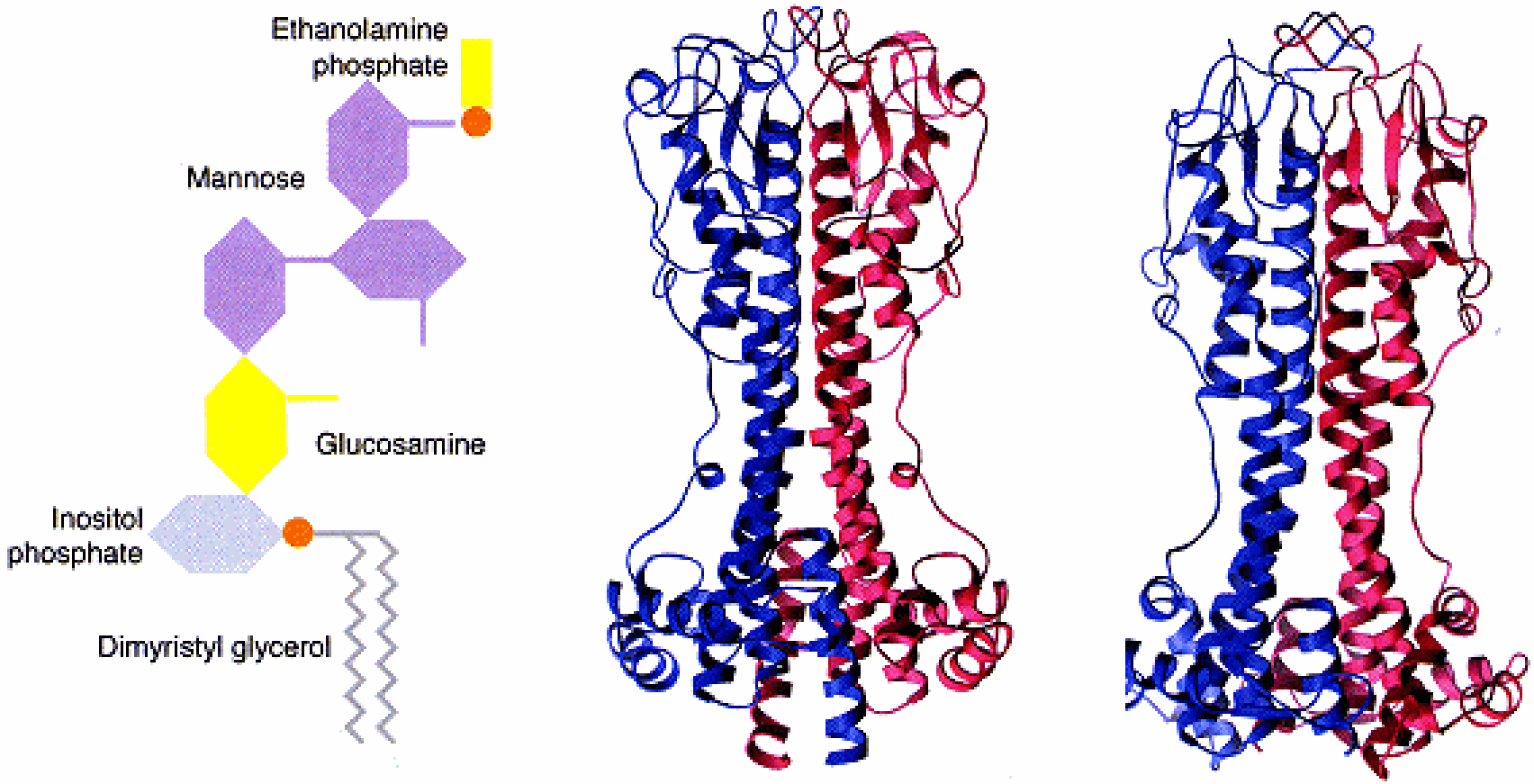
The VSG (variant surface glycoproteins) molecules are present on the surface of the trypomastigote as a 12-25 nm thick coat; each has a molecular size of 60kD with ~500 amino acids and 20 monosaccharide units.

George Cross
Rockefeller University

T. brucei Variant Surface Glycoprotein



20nm x 20nm
section of membrane



COLOR PLATE 2 (See also Figure 5.2) (Left) Structure of the minimal ('core') VSG GPI anchor and (right) three-dimensional structures of the amino-terminal domains of VSG MIT at 1.2 (left) and ILT at 1.24 (right). The dark and light ribbons indicate the atomic traces of the two identical units comprising the dimeric domain. The amino termini are at the top of the molecule, which forms the outer face of the coat. The bottom of this domain is linked to the carboxy-terminal domain, for which a structure is not available, which is then linked to the GPI anchor. The image was kindly provided by Ms Lore Leighton.

The VSG coats are shed on differentiation from the bloodstream trypomastigotes to the insect procyclics. The procyclic forms are covered with two type of glycoproteins (procyclins)

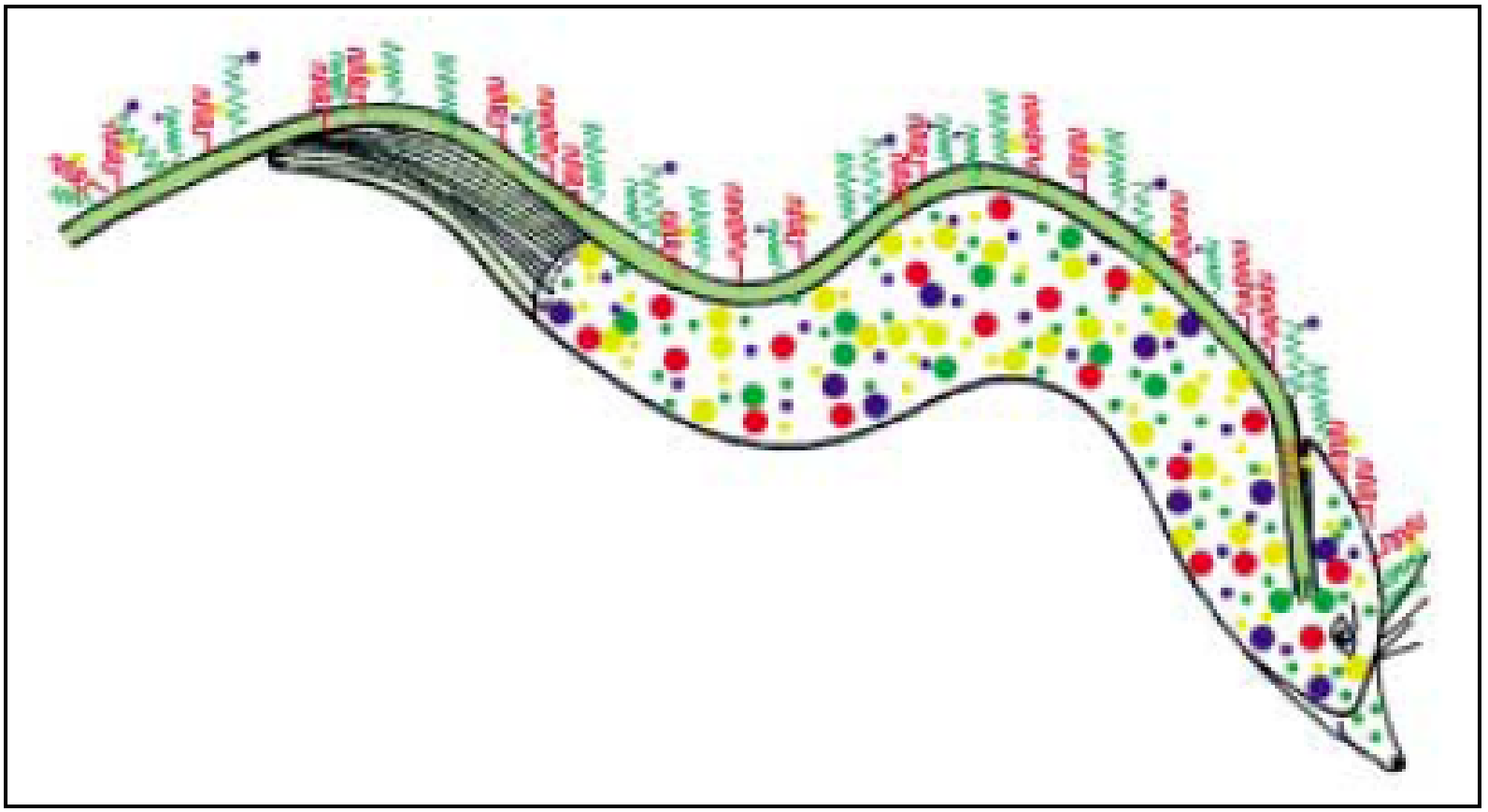


Fig. 1. The 'Spiny Norman' model of procyclins expressed on the surface of *Trypanosoma brucei* procyclic or epimastigote forms. This 'coat of many colours' represents the different procyclin isoforms expressed during development of the parasites in the tsetse vector. Red surface molecules are the EP procyclins showing varying EP repeat lengths with yellow N-linked carbohydrates. Green surface molecules are the GPEET procyclins with blue phosphate groups attached to threonine residues. Only part of the coat is shown but in reality, the entire surface is covered. At various times during development in the fly vector, only some or all of the procyclin isoforms will be expressed. For image clarity, the proposed glycocalyx formed by the glycosylphosphatidylinositol (GPI) anchor carbohydrates[14] is not shown.

Transcription in eukaryotic cells

RNA Polymerase I – Transcribes ribosomal RNA (in nucleolus)

RNA Polymerase II – Transcribes mRNA, most snRNAs and microRNAs

RNA Polymerase III – Transcribes 5S rRNA, U6 splicesosomal RNA and tRNAs

Trypanosomes are the exception!

Previous work showed that transcription of the VSG genes and the procyclin genes is inhibited by α – amanitin, a specific inhibitor of Pol I transcription.

But this was indirect evidence.

In 2003 Arthur Gunzl provided definitive proof that Pol 1 transcribes VSG genes, the GPEET procyclin genes in addition to rRNA genes.

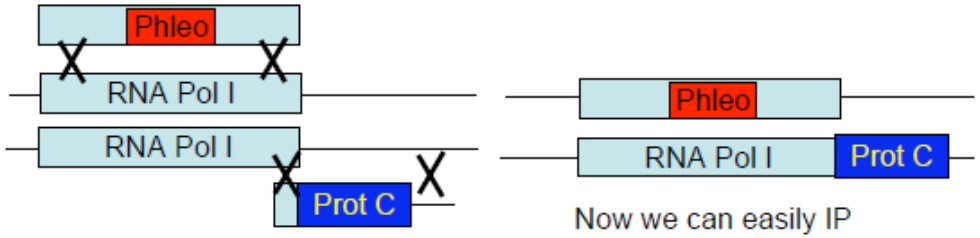
Gunzl generated a *T. brucei* cell line which exclusively expresses protein C epitope-tagged RNA pol I.

Using an anti-protein C immunoaffinity matrix, he specifically depleted RNA pol I from transcriptionally active cell extracts.

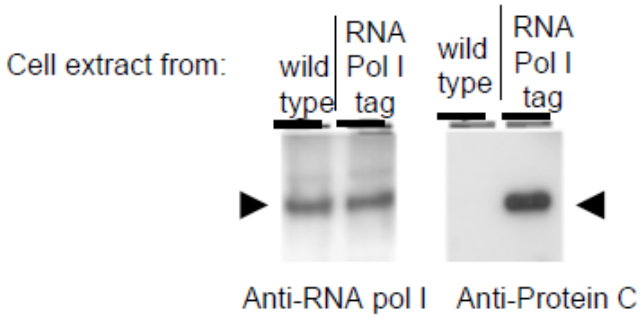
The depletion of RNA pol I impaired *in vitro* transcription initiated at the rDNA promoter, the GPEET procyclin gene promoter, and a VSG gene expression site promoter but did not affect transcription from the spliced leader (SL) RNA gene promoter, which is Pol II mediated.

Epitope Tagging

Adding a sequence coding a peptide fragment that you already have Ab for.Protein C, HA, c-Myc, Strep, Flag, BB2….the list goes on and on.

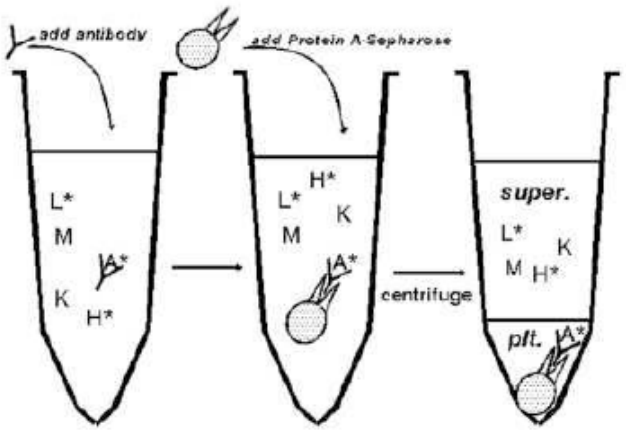


Now we can easily IP and detect RNA Pol I

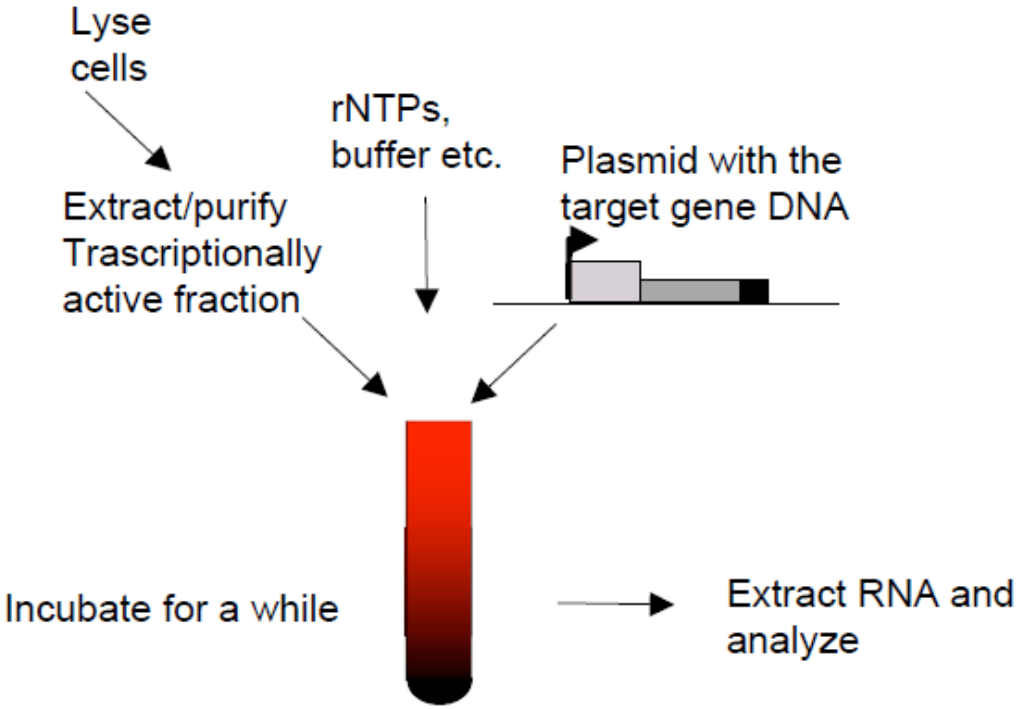


Immunoprecipitation

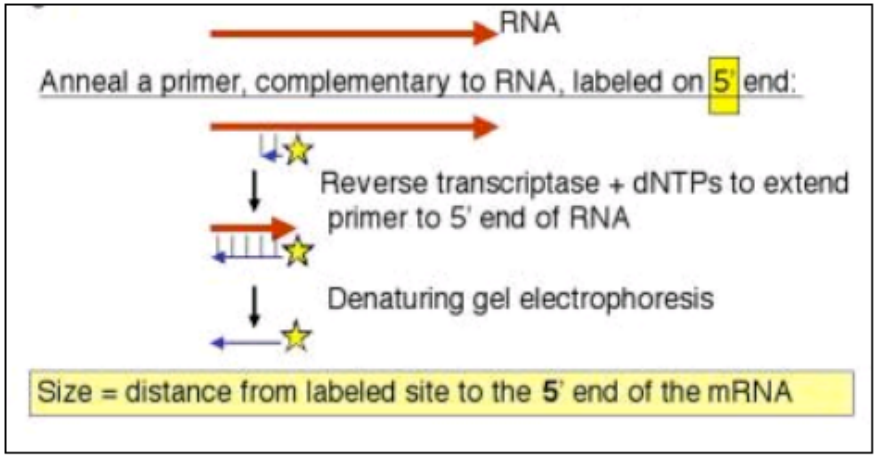
Protein A binds the Fc region of Abs



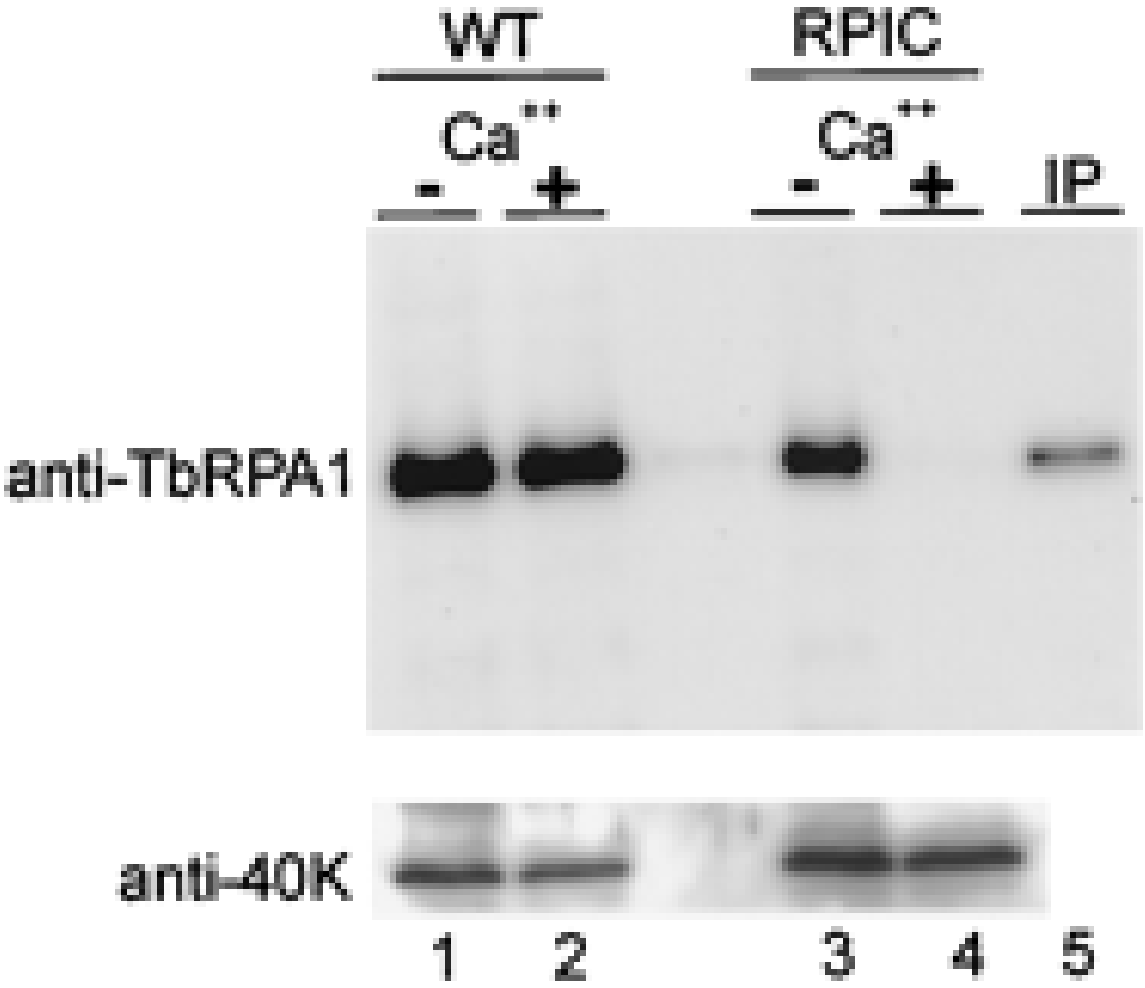
In vitro Transcription



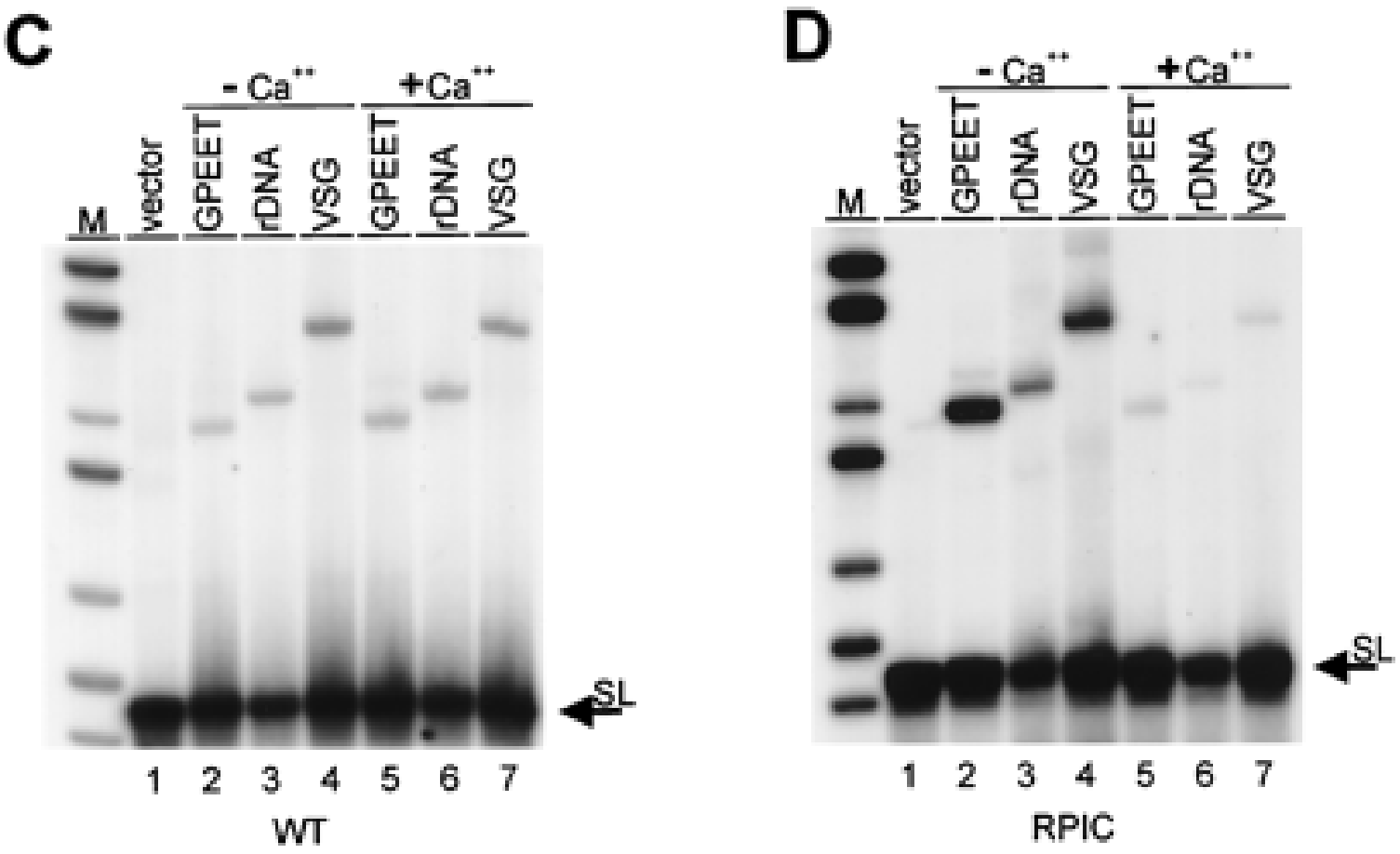
Primer Extension – measures RNA quantity



The tagged Pol I subunit was removed from the cell extract by immunodepletion, using an antibody against the protein C epitope (requires Ca++)

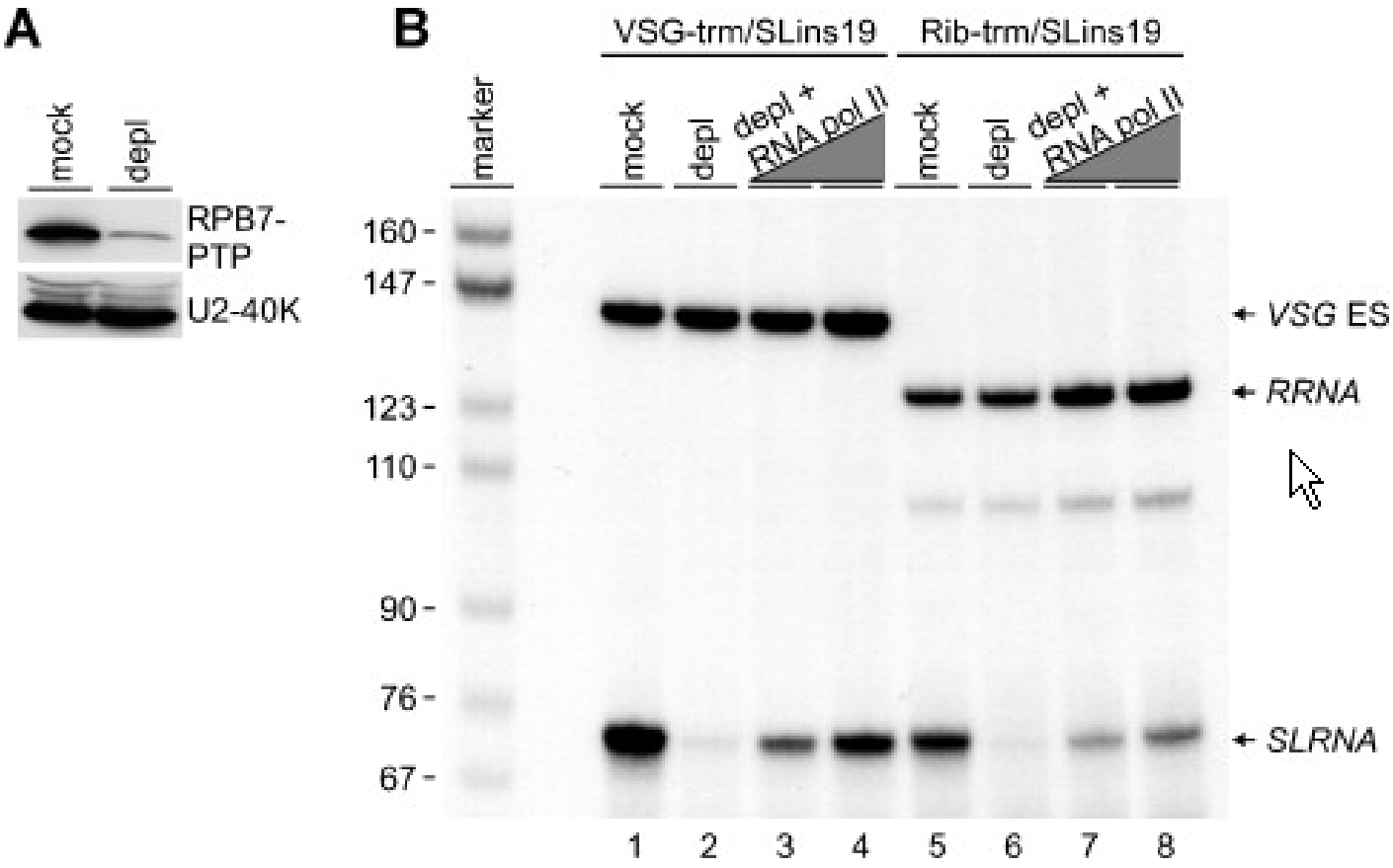


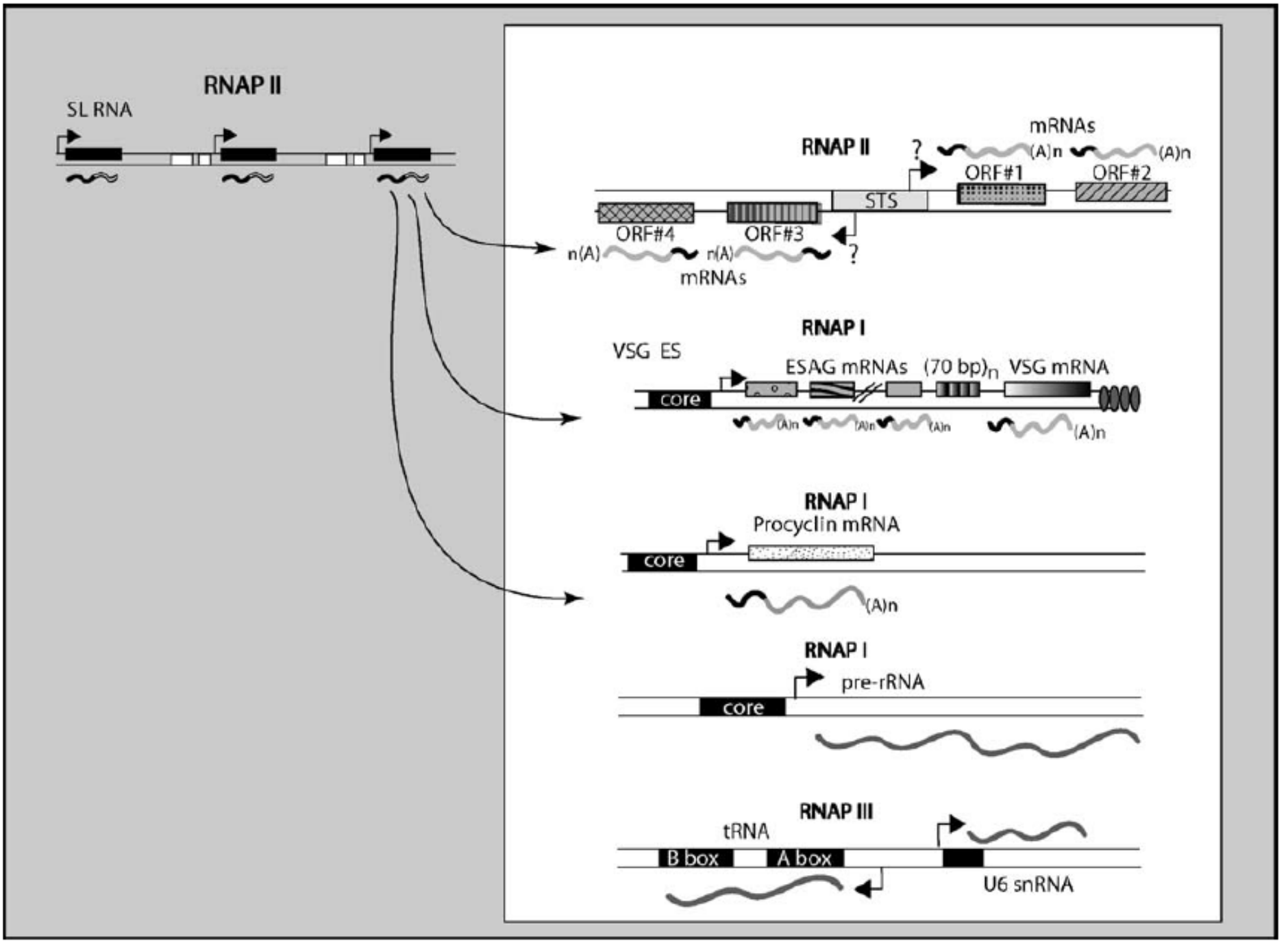
The extracts without the Pol I did not transcribe the GREET procyclic, rRNA and VSG genes, but did transcribe the SL genes



In 2011 Gunzl showed that immuno-depletion of RPB7, a subunit of *T. brucei* RNA Pol II, abolished Pol II transcription, but had no effect on rRNA or VSG expression site promoter Pol I transcription.

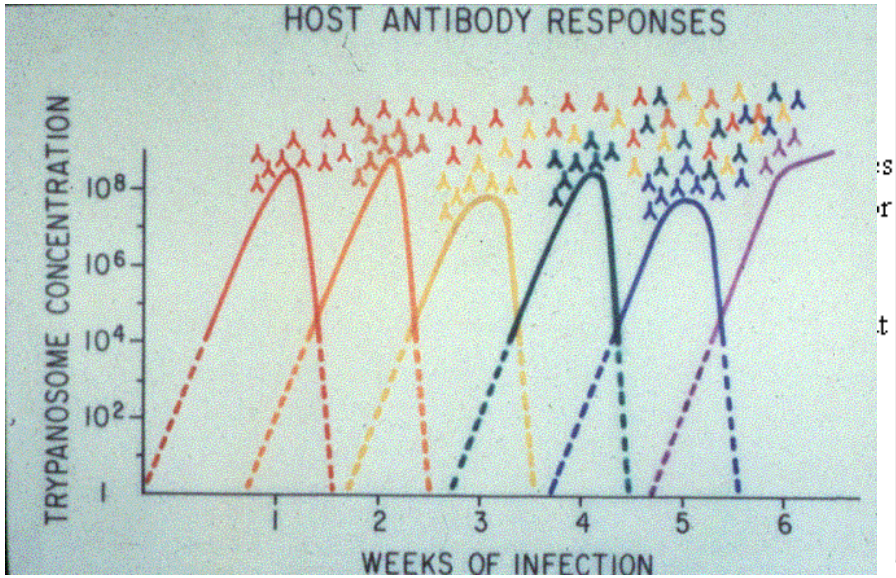
He generated a cell line in which RPB7 was tagged with the PTB epitope tag. This was used to immuno-deplete RPB7 from a cell extract which was then used for *in vitro* transcription. Transcription was assayed by primer extension of labeled oligos specific for each gene.

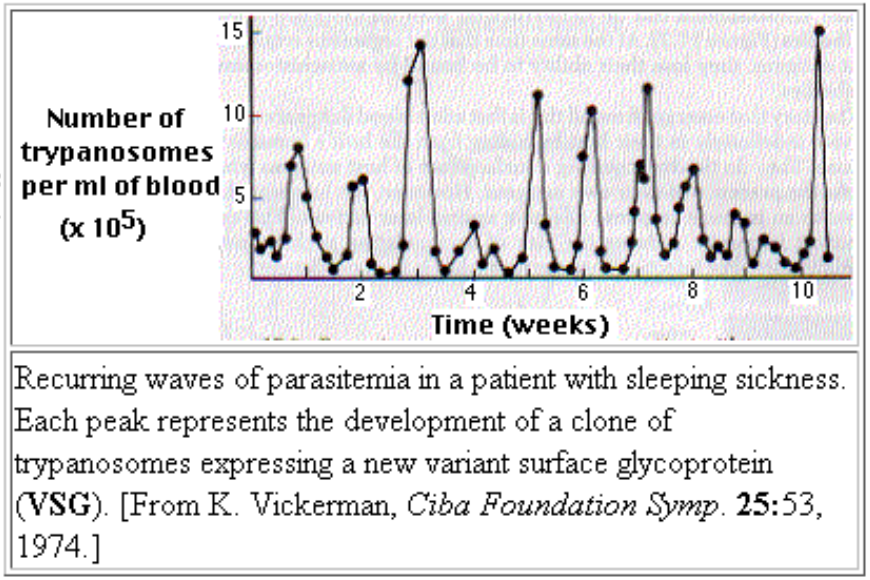



RNAP II
SL RNA
RNAP II
mRNAs
(A)n
(A)n
?
ORF#1
ORF#2
STS
ORF#4
ORF#3
n(A)
n(A)
?
mRNAs
RNAP I
VSG ES
ESAG mRNAs
(70 bp)n
VSG mRNA
core
(A)n
(A)n
(A)n
(A)n
RNAP I
Procyclin mRNA
core
(A)n
RNAP I
pre-rRNA
core
RNAP III
tRNA
B box
A box
U6 snRNA

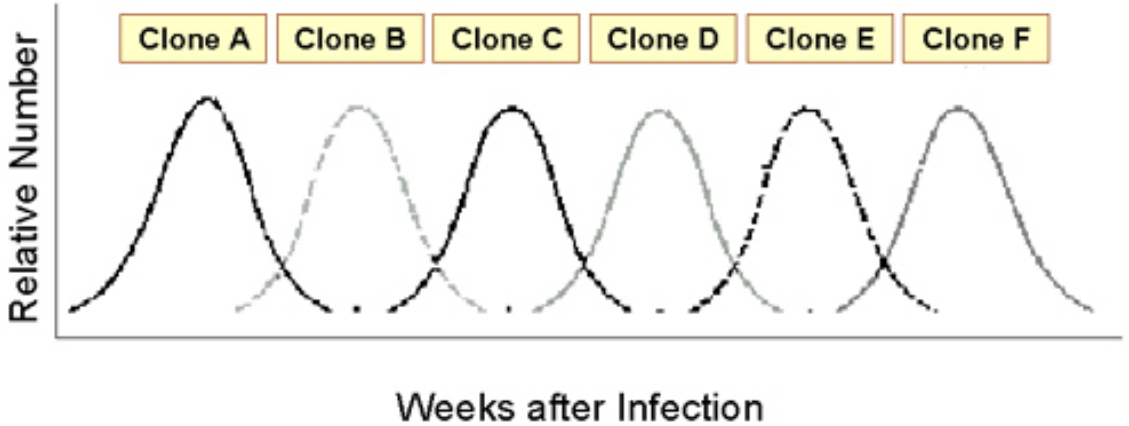
ANTIGENIC VARIATION IN AFRICAN TRYPANOSOMES

As early as 1910 Ross and Thompson found that during the course of a *T. brucei* infection there were a series of relapsing parasitemias. When these relapse forms were injected into rabbits so that an antiserum was produced it was shown that each relapse form differed antigenically from the others. Human trypanosomiasis shows same relapsing parasitemias





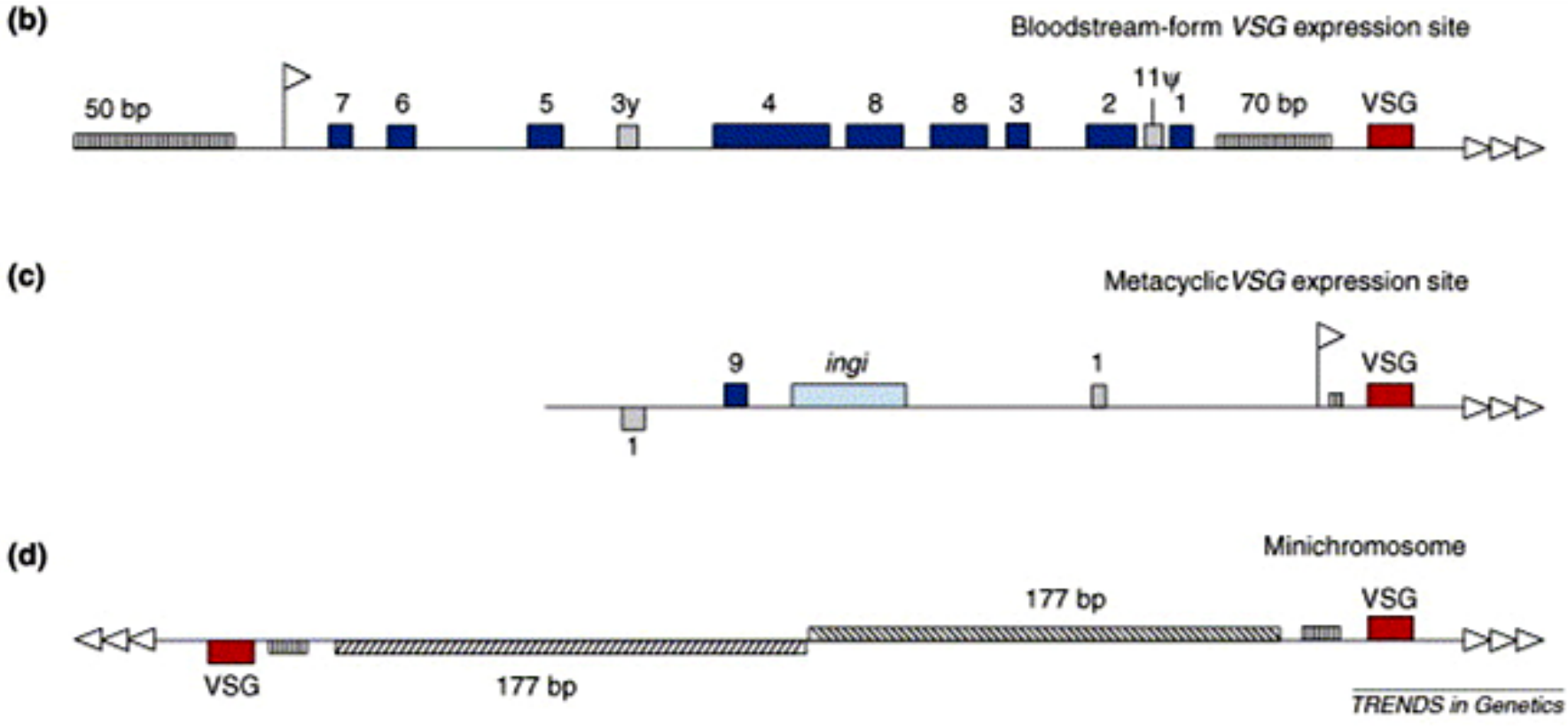
Recurring waves of parasitemia in a patient with sleeping sickness. Each peak represents the development of a clone of trypanosomes expressing a new variant surface glycoprotein (**VSG**). [From K. Vickerman, *Ciba Foundation Symp.* **25:**53, 1974.]

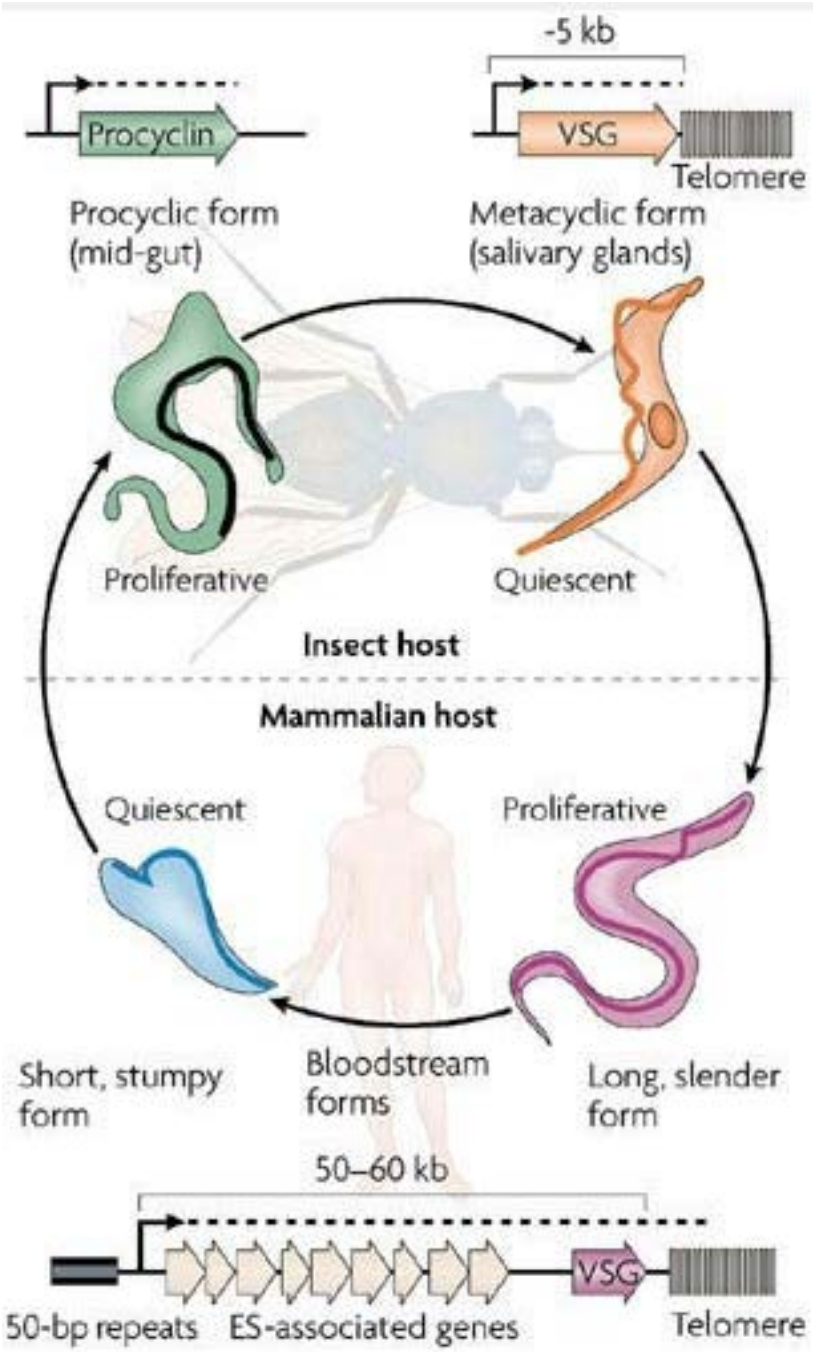


Telomeric Expression Sites

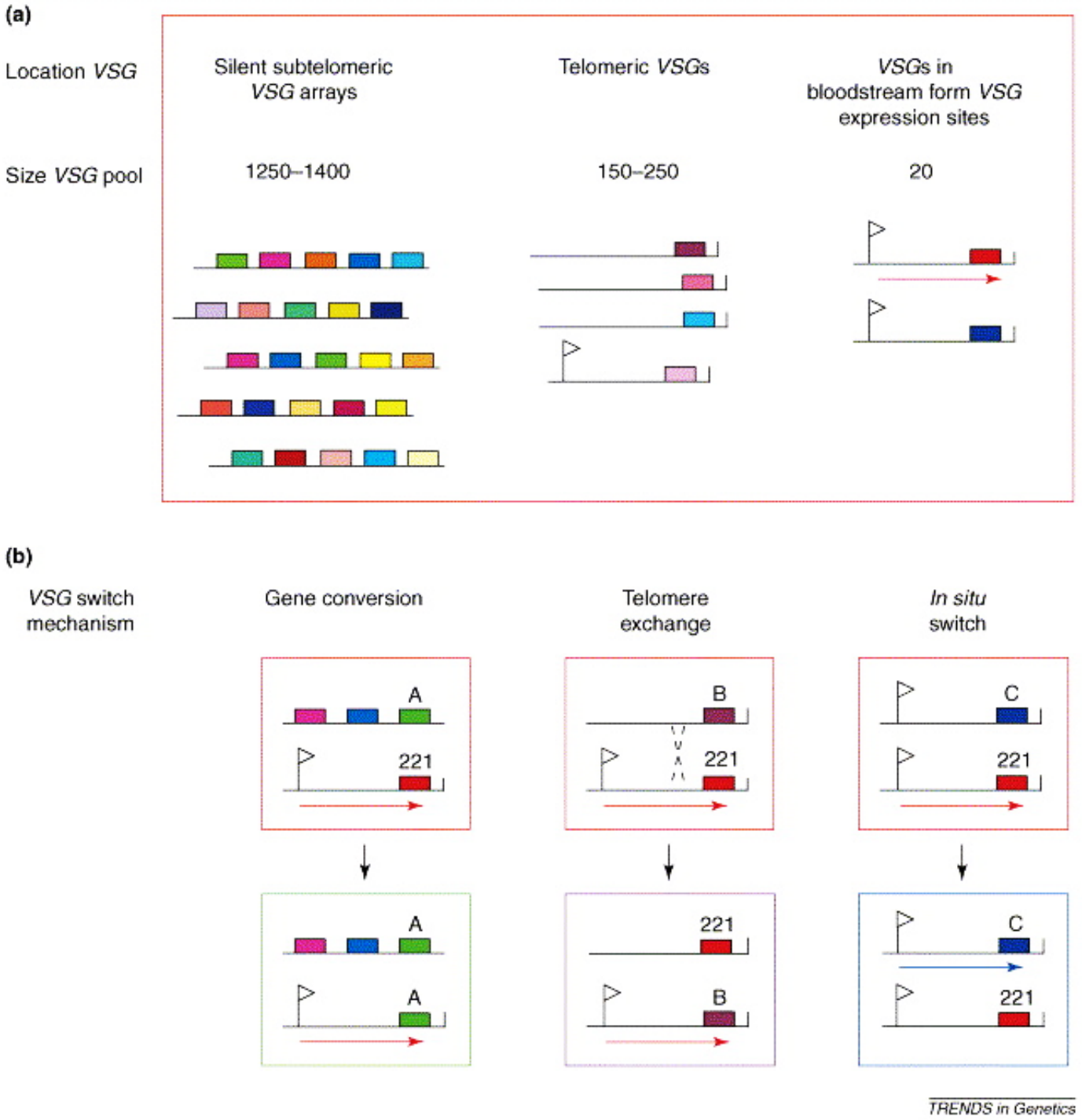
Often, there is a new copy of the expressed gene in the next antigenic variant. This is termed the Expression-Linked Copy.

There are a small number of different telomeric expression sites. Only one expression site is actively transcribed at one time.




-5 kb
Procyclin
VSG
Telomere
Procyclic form
(mid-gut)
Metacyclic form
(salivary glands)
Proliferative
Quiescent
Insect host
Mammalian host
Quiescent
Proliferative
Short, stumpy
form
Bloodstream
forms
Long, slender
form
50–60 kb
VSG
50-bp repeats
ES-associated genes
Telomere

Piet Borst


(a)
Location *VSG*
Silent subtelomeric *VSG* arrays
Telomeric *VSG*s
*VSG*s in bloodstream form *VSG* expression sites
Size *VSG* pool
1250–1400
150–250
20
(b)
VSG switch mechanism
Gene conversion
Telomere exchange
In situ switch
A
221
B
C
TRENDS in Genetics

Sequencing VSG Expression Sites


Gloria Rudenko

1. Telomeric sequences are difficult to clone and so are underrepresented in the genome database.
2. Rudenko was able to clone ES-containing telomeres using yeast artifical chromosomes. Did not clone the metacylcic ES repertoire.

Results:

1. BES have remarkably conserved gene order.

2. 14 distinct BES in *T. brucei* strain 427, 23 BES in *T. brucei* strain EATRO 2340, 13 BES in *T. gambienese*, 16 BES in *T. equiperdum*.

3. Some ESAG duplications and truncations observed. Many examples of VSG and ESAG pseudogenes - especially ESAG 3 and ESAG11.

4. Most ESAGs are dispensable.

5. No correlation between BES number and host range.

Summary of the number and predicted function of *ESAG*s in the bloodstream *VSG* expression sites of *T. b. brucei* Lister 427

ESAG	Number of *T. b. brucei* Lister 427 BESs that contain specified ESAG[a]	Function or properties	Refs
1	11	Membrane glycoprotein	[46]
2	11	GPI-anchored glycoprotein?	[36]
3	13[a]	Membrane glycoprotein?	[36]
4	10[c]	Receptor-like adenylate cyclase	[47]
5	13[d]	Lipid transfer/lipopolysaccharide binding?	[43]
6	13	Transferrin receptor subunit	[38]
7	13	Transferrin receptor subunit	[38]
8	10[e]	Nucleolar RNA stability regulator?	[48]
9	1	Unknown	[49]
10	7	Biopterin transporter?	[50]
11	11[f]	GPI-anchored glycoprotein?	[51]
12	5	Unknown	[17]
SRA	0[g]	Resistance to human serum	[28]

[b] Six BESs contain only *ESAG3* pseudogenes.

[a] From a total repertoire predicted to comprise 14 BESs.

[c] Two BESs contain only *ESAG4* pseudogenes.

[d] Two BESs contain only *ESAG5* pseudogenes.

[e] Found downstream of a duplicated BES promoter.

[f] Only *ESAG11* pseudogenes detected.

[g] Only described in *T. b. rhodesiense*.

Revelations from the *T. brucei* genome sequence

The astonishing view revealed by the sequence of the *T. brucei* genome is that the vast majority of the silent *VSG* genes within the *VSG* arrays present on *T. brucei* megabase chromosomes are pseudogenes.

Of the 806 *VSG* genes analysed in the fully assembled parts of the *T. brucei* 927 genome sequence, only 7% seem to be intact and containing all recognizable features of known functional *VSG*s. By contrast, 66% of the *VSG*s are full-length pseudogenes with frameshifts or in-frame stop codons, 18% of the *VSG*s are gene fragments, mainly containing *VSG* 3′ ends, and 9% of the *VSG*s are atypical in some way, in that they lack features that would be expected on functional *VSG*s .

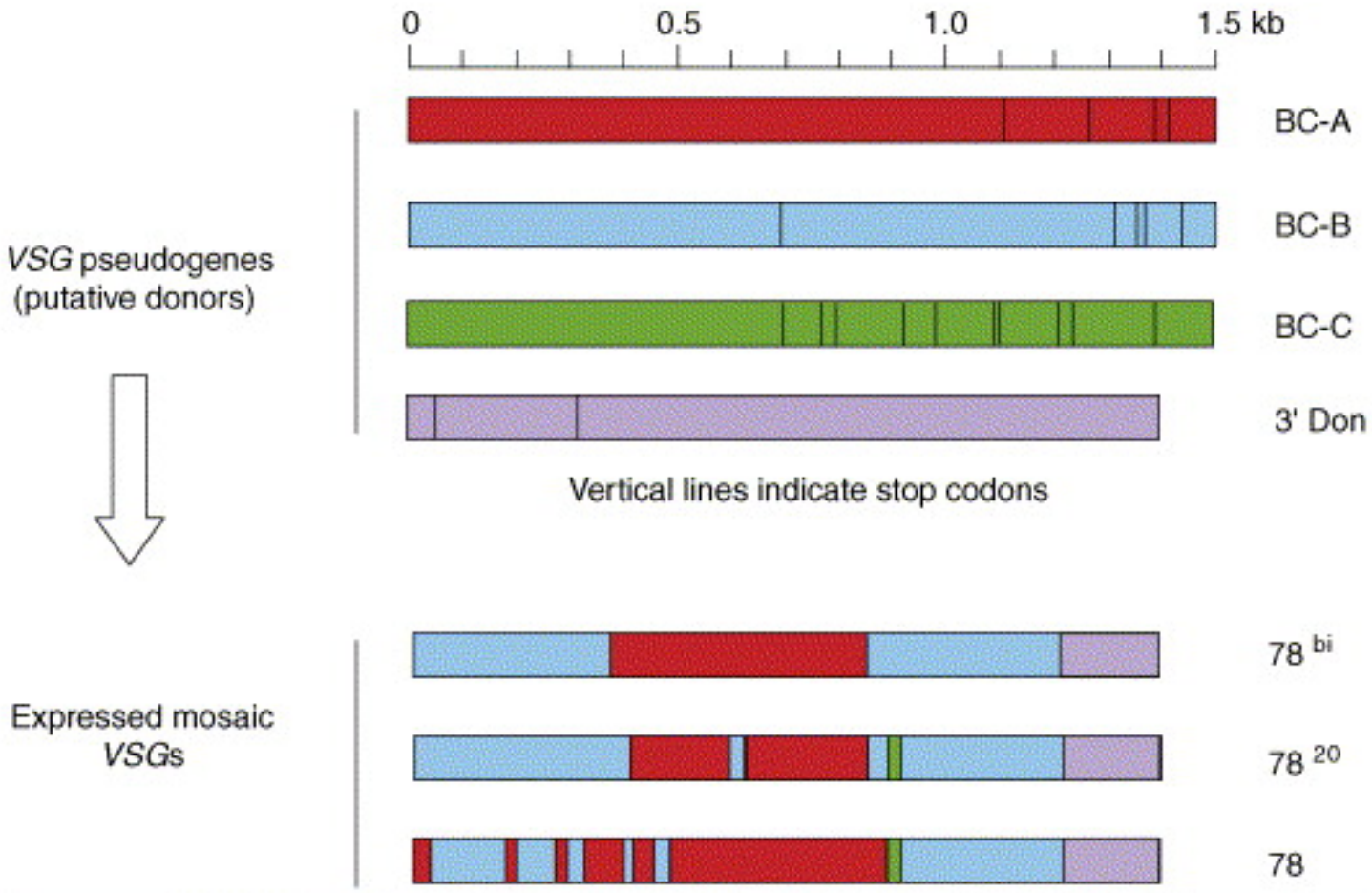
A second surprising feature of the *T. brucei* genome sequence is that all of these *VSG* genes are present in arrays ranging in size from three to 250 copies, which are mainly located at subtelomeres. The majority of the *VSG* genes analysed have a strand orientation pointing away from the telomere repeats, unlike the *VSG*s present within *VSG* expression site transcription units, which point in the direction of the telomere repeats.

How could all of this *VSG* gene degeneracy have been missed for so long?

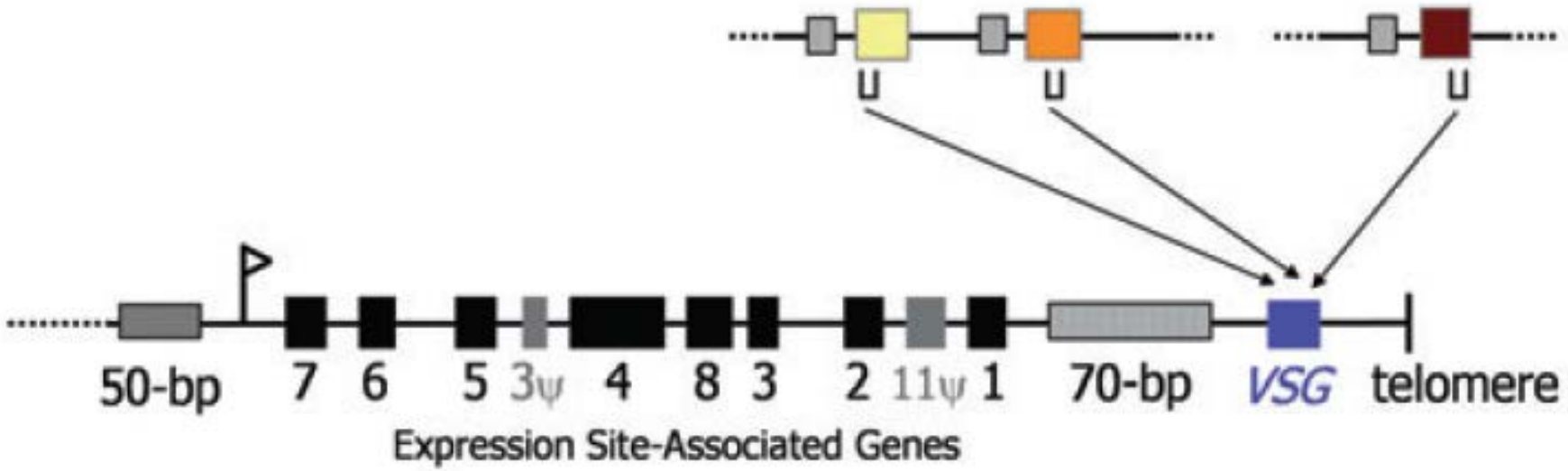
Virtually all researchers studying antigenic variation in African trypanosomes have for technical reasons concentrated on VSG switches occurring relatively early in an infection. Typically, single relapses (or switches away from one *VSG* gene) are analysed. Analysis of VSG switch events occurring during chronic infections is complicated by the fact that it can be impossible to determine whether a switch variant has arisen from a previous antigenic peak, or was already present as a minor variant within the infection. One consequence of this experimental bias for the analysis of early VSG switch events has been to concentrate attention on the most frequent VSG switch events, and on that portion of the *VSG* repertoire composed of intact genes most likely to be activated early in infection.

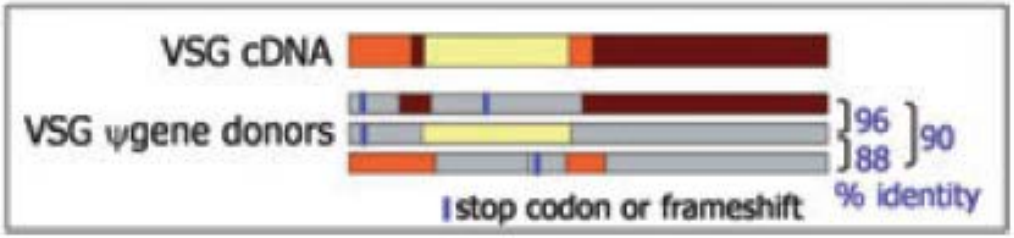
VSG switching mediated by "segmental" gene conversion

Dave Barry



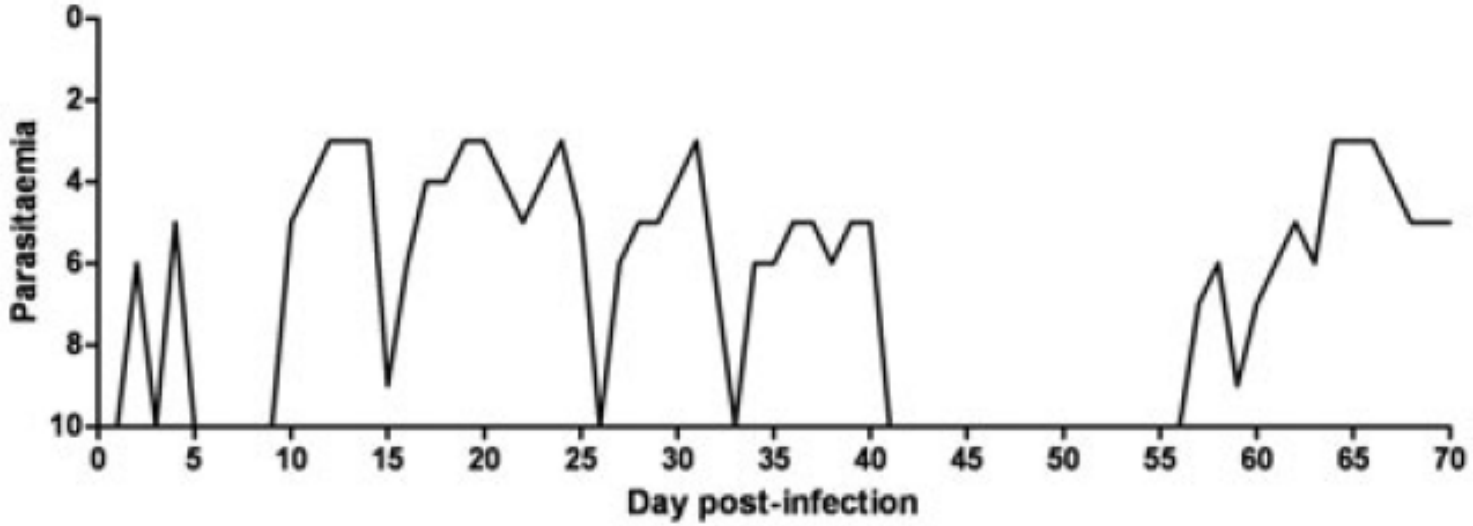
Mosaic VSG genes made from segments of the VSG pseudogenes are expressed late in the infection

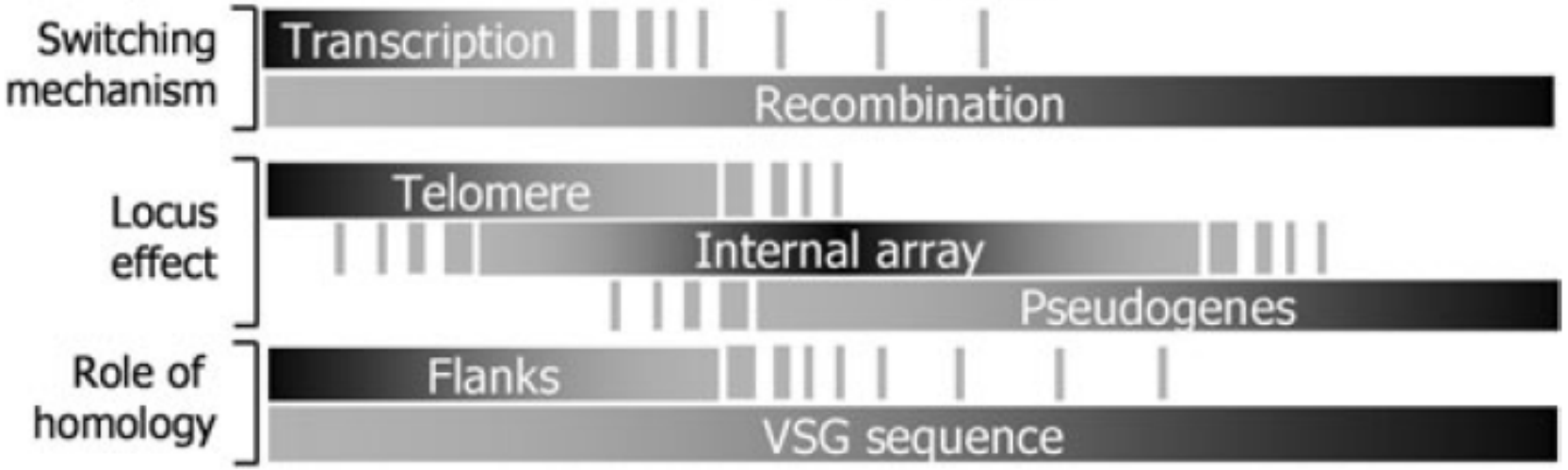




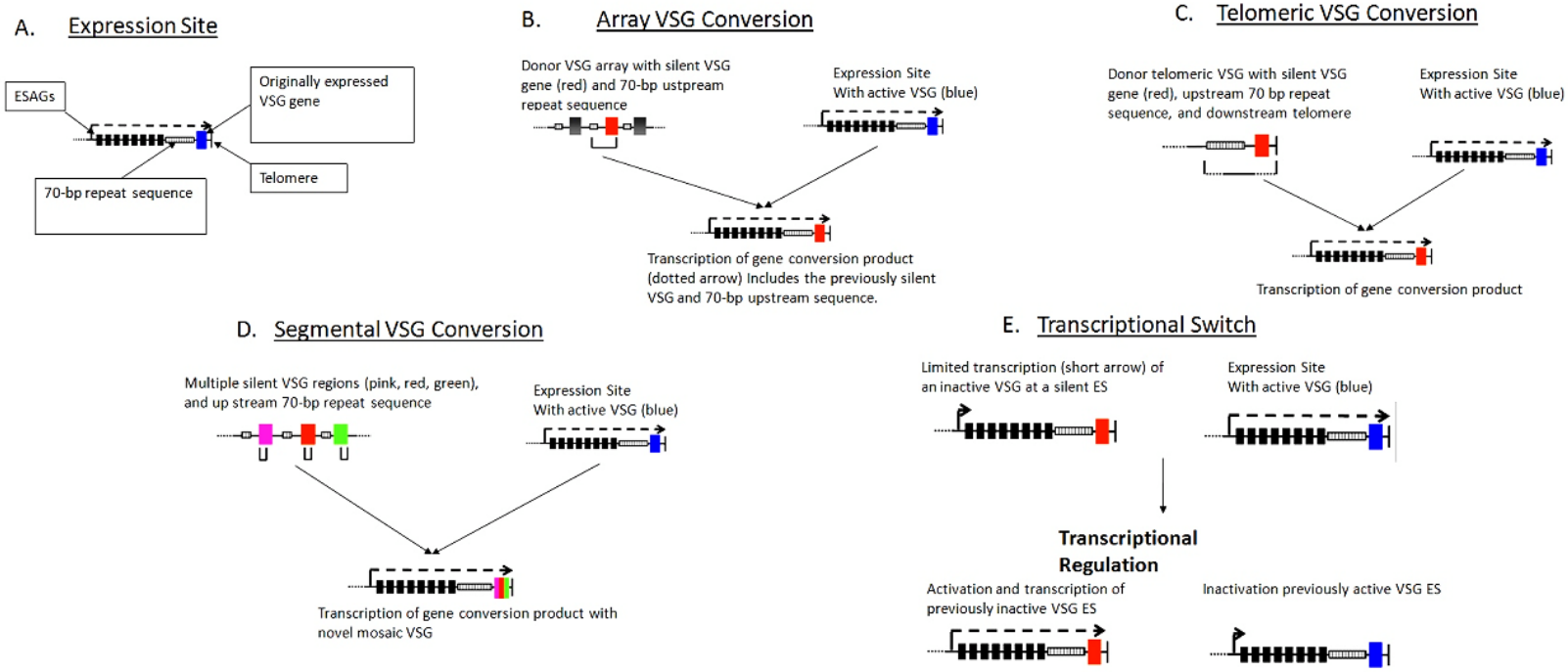
VSG switching hierarchy in *T. brucei*

Parasitemia in infected cow





Mechanisms of VSG Switching in T. brucei



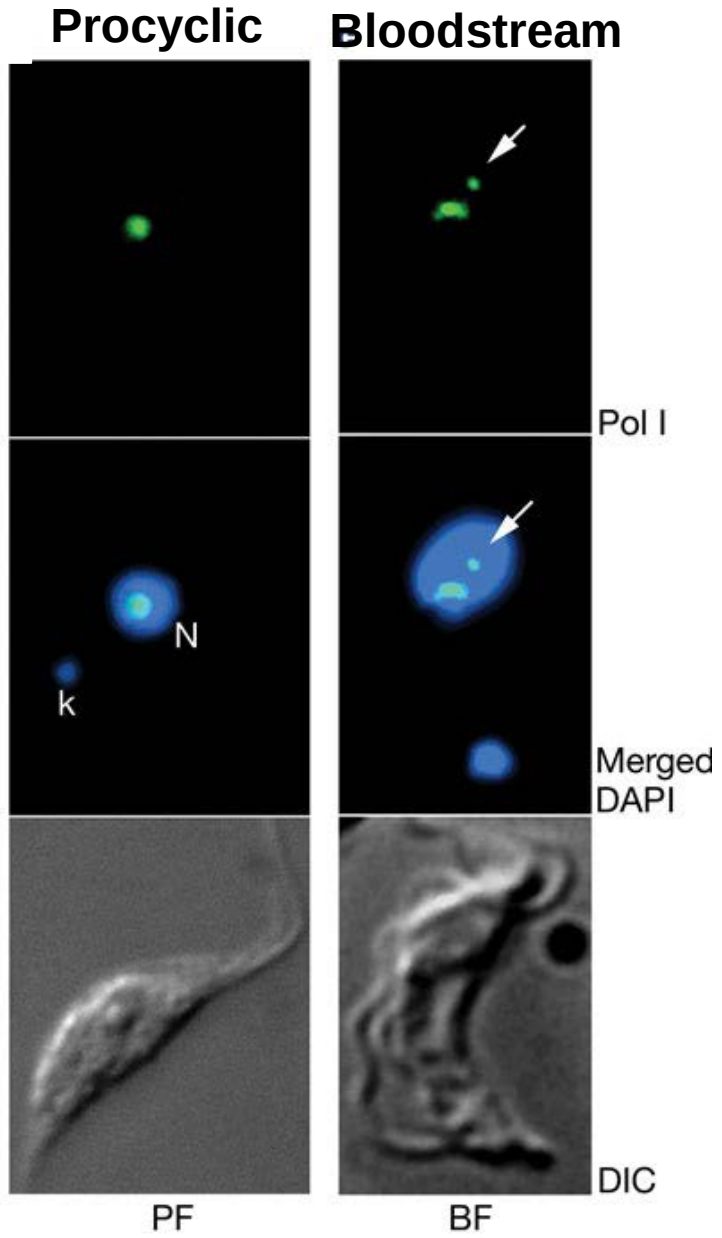
(A) Structure of the expression site including the expression site associated genes (ESAG), 70 base pair repeat up-stream sequence, expressed VSG gene, and the telomere. (B) Mechanism of array VSG conversion: A silent VSG is copied from a subtelomeric VSG array into an ES, where it replaces the active VSG. (C) Telomeric VSG conversion: A telomeric VSG (including 70 bp repeat sequence upstream and telomere downstream) replaces the active VSG in the ES (D) Segmental VSG conversion: Sequence is copied from multiple inactive VSG genes and combined into a novel mosaic VSG that occupies the ES. (E) Transcriptional VSG switching: A non-recombination based mechanism that activates a new (previously silent) ES, while inactivating the previously active ES.

Localization of Pol I VSG transcription leads to a model

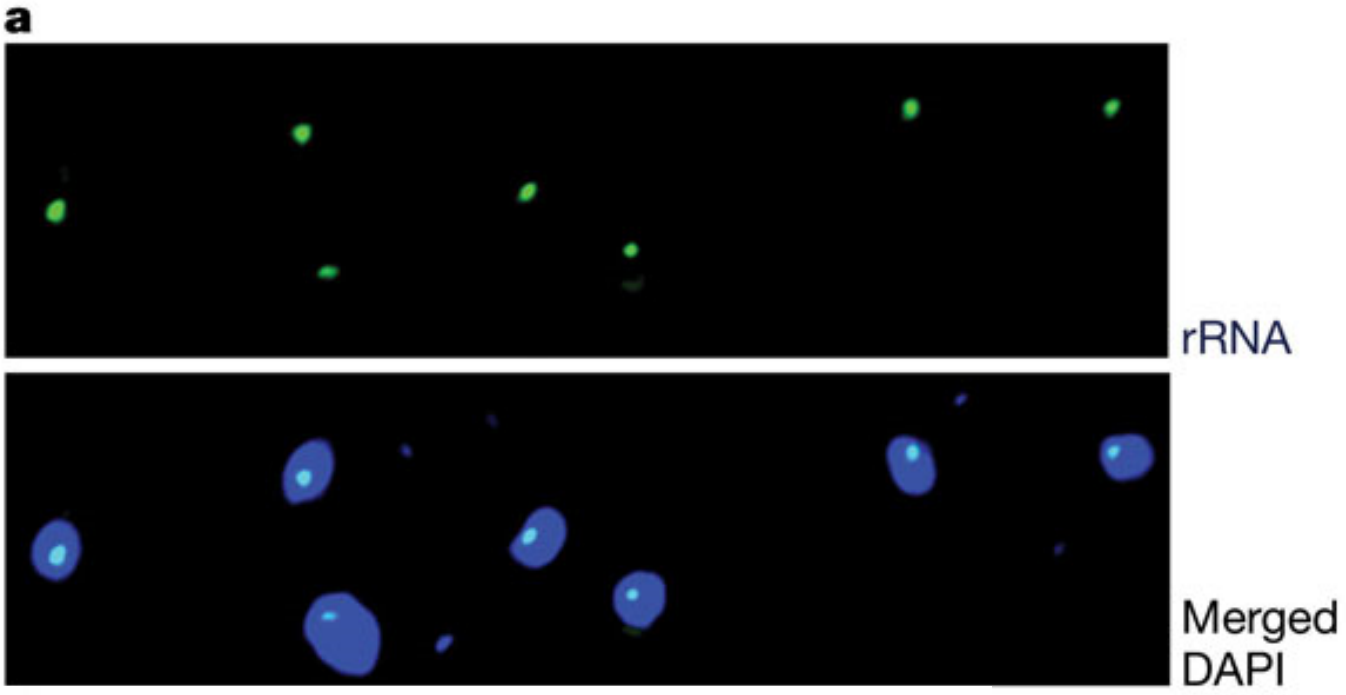
Identification of extranucleolar body containing Pol I in BS *T. brucei*

Immunofluorescence using antibody against Pol I large subunit.

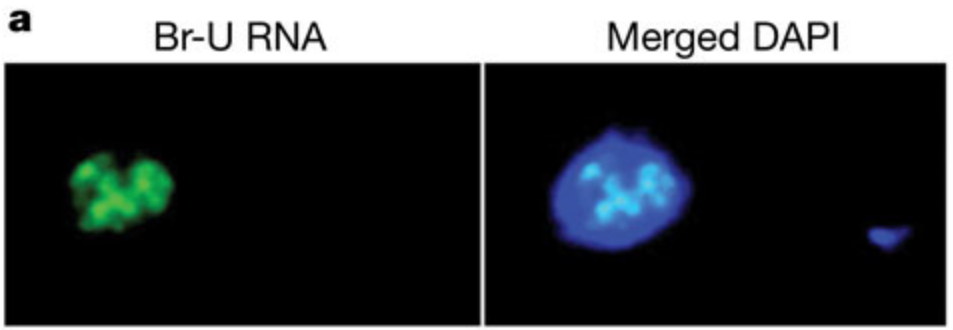
Keith Gull



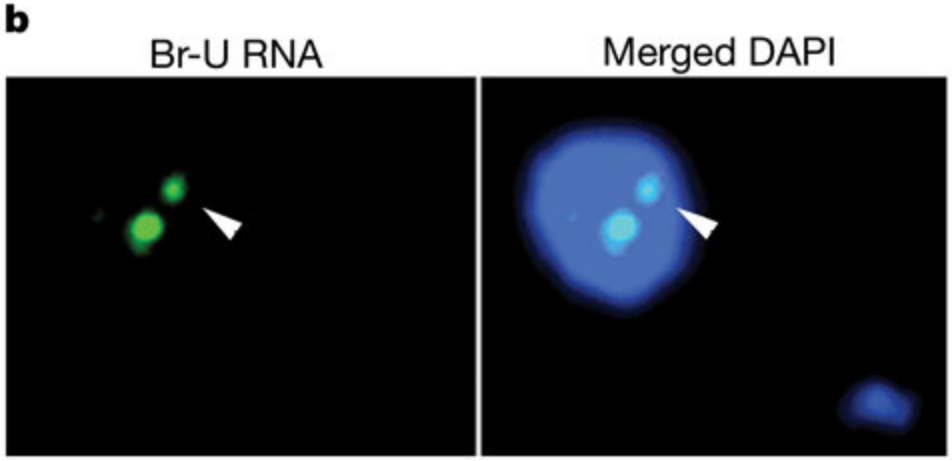
The pol I body does not contain rRNA



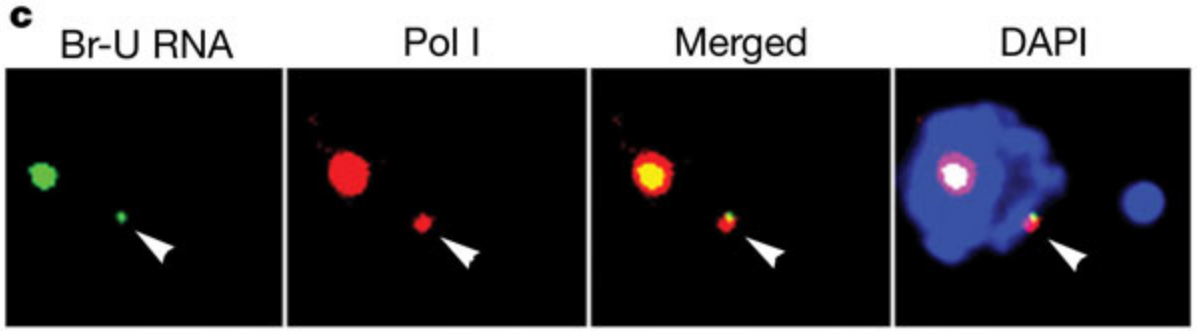
Total transcription, as revealed by Br-UTP (Br-U) incorporation foci (green) using a monoclonal antibody reactive to B-dUTP



Two transcriptional foci detected in the presence of α-amanitin



Colocalization of the α-amanitin-resistant nascent RNA Br-UTP-labelled foci (green) with the nucleolar and extranucleolar body



Model:

ESB-dependent ES recruitment leads to the activation of a single ES and inactive ES's are excluded from this body.

In situ switching is mediated by a process in which attachment of the active ES becomes unstable, allowing occupancy by an inactive ES.

Sexual phase in African trypanosome life cycle

Wendy Gibson

Initially people used classical genetics by analysis of progeny after Passage through the tsetse fly. Controversial.

In 1993 Gibson used two drug resistence markers introduced into *T. brucei t*o show appearance of hybrids after passage through the tsetse fly.

In 2008 she definitively established and visualized a sexual phase by using two flourescent markers.Transfected trypanosomes with Green Fluorescent Protein (GFP) or Red Fluorescent Protein (RFP).

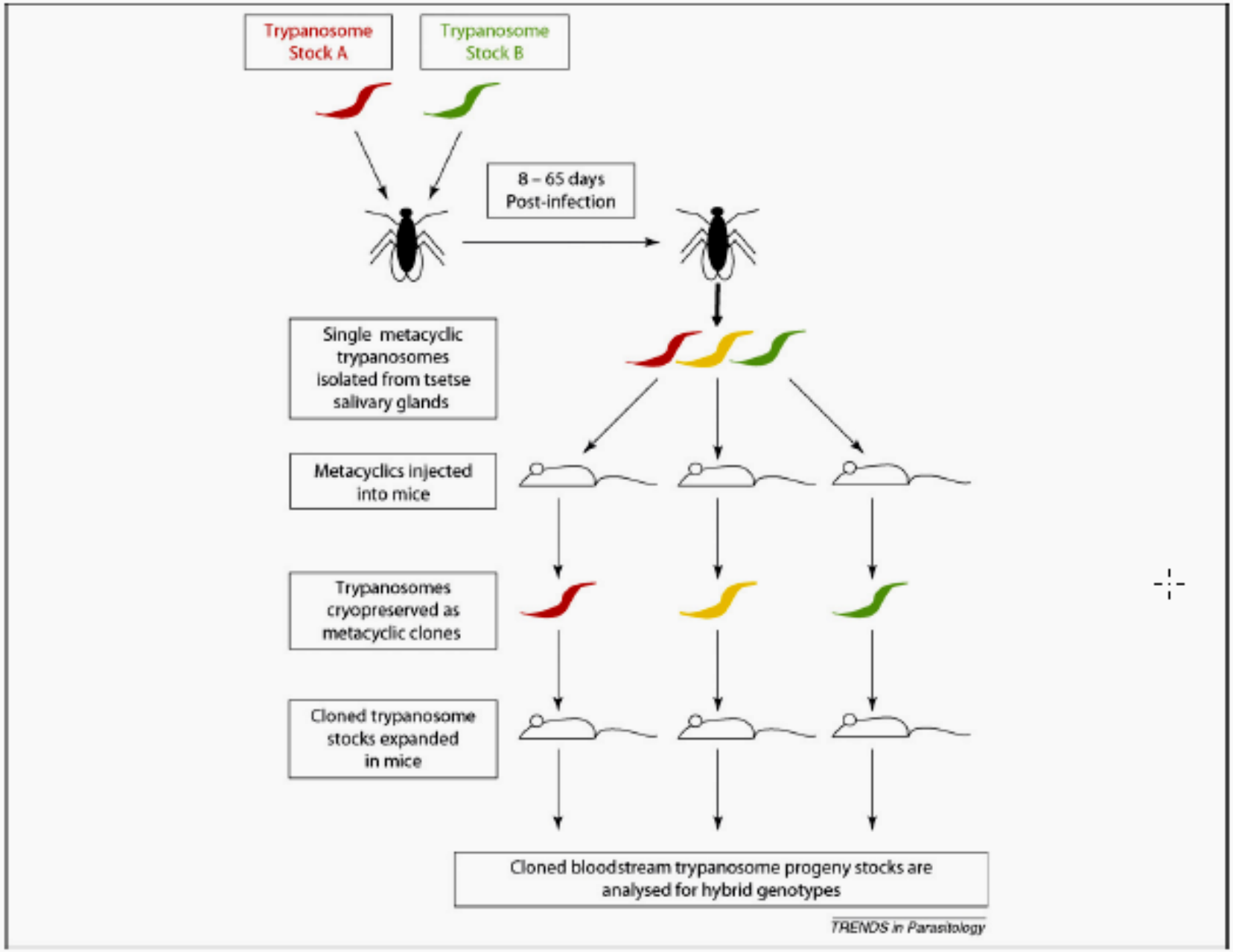
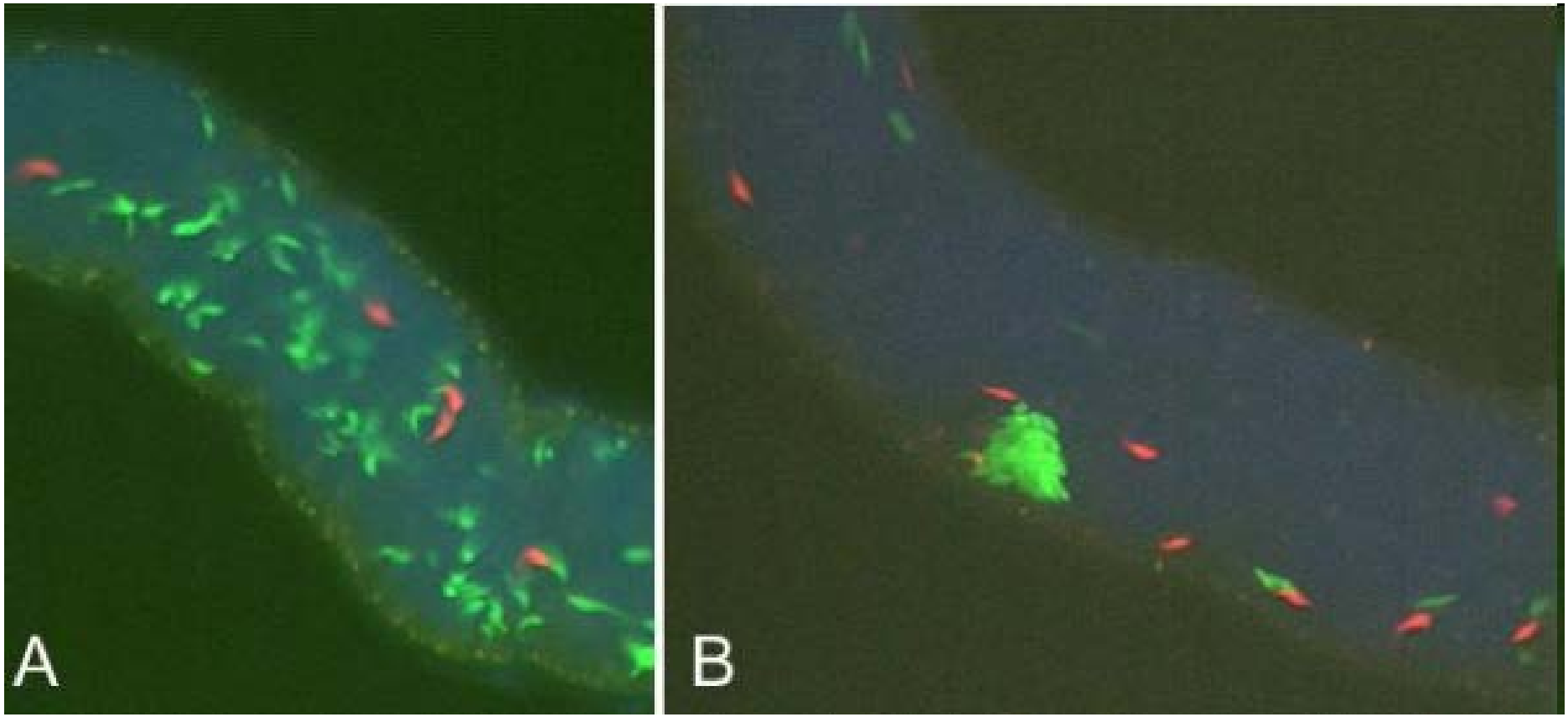
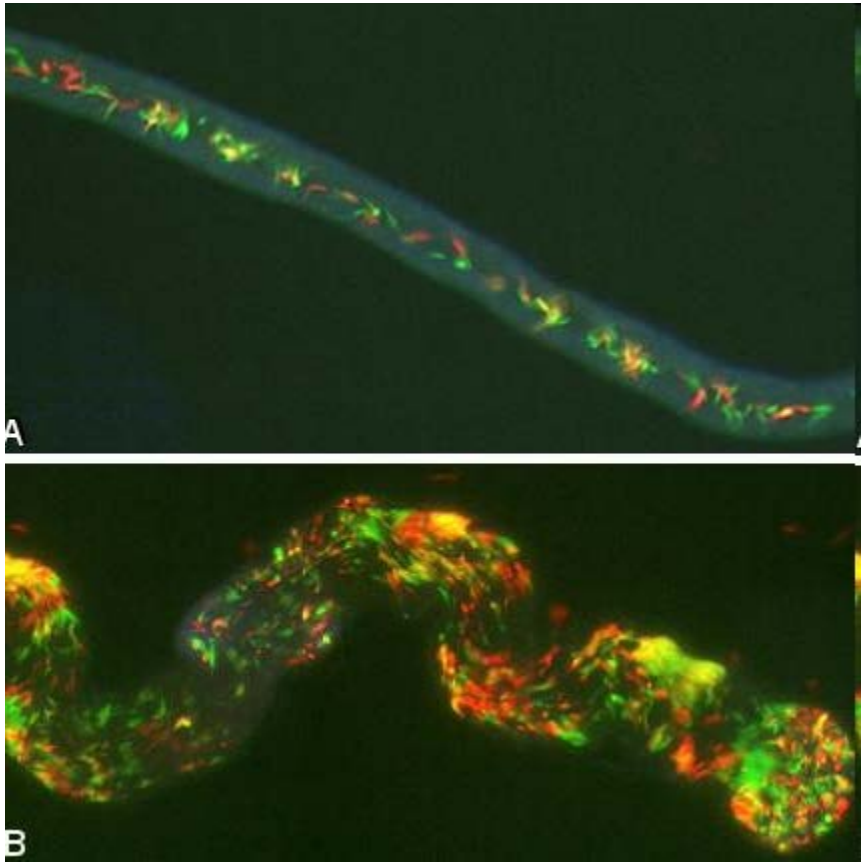


Figure 1. Experimental genetic crosses in *Trypanosoma brucei*. This schematic shows the procedure for carrying out genetic crosses in *Trypanosoma brucei*. Two parental stocks are mixed by feeding tsetse flies with infected blood that contains both parents. Tsetse are maintained and, at varying time points between eight and 65 days, are dissected to isolate single metacyclic trypanosomes. These single trypanosomes are further expanded by growth in mice and then cryopreserved. Progeny clones are then resurrected by expansion in mice, followed by DNA extraction for analysis by a variety of genetic techniques.

Close proximity of red and green trypanosomes in salivary glands at early establishment. Flies dissected at A. 20 days and B. 15 days after infection.



Yellow hybrids in salivary glands.



Dissected salivary glands with mixed infection of red, green and yellow trypanosomes. A. Salivary duct from 27 day infection. B. Portion of salivary gland showing blind end from 20 day infection.

Conclusions: The short epimastigotes that colonize the salivary glands mate and then rapidly populate the glands. No intermediate forms seen. A high percentage of polyploid cells. Live cells.